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CYTOLOGICAL STUDIES IN MEDICAGO, MELILOTUS AND TRIGONELLA[1]

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Abstract

The investigation reported herein is an effort to secure information through the use of cytological methods, which may assist in providing a better understanding of the true relationships of species and varieties within the genus *Medicago*, and to compare cytologically, in a general way, this genus with the related genera *Melilotus* and *Trigonella*. In order to emphasize the present unsatisfactory taxonomic situation the morphological characters of the three genera are described and the taxonomic classifications of the species for each is reproduced as presented by Taubert (49). Classifications for *Medicago* by Dr. Candolle (11) and by Hegi (15) are also outlined for purposes of drawing attention to certain discrepancies between the classifications of *Medicago* by Taubert and by Hegi. Reference is made to specific taxonomic difficulties in *Medicago*. The general distributions of the three genera are given.

In the section of the paper on "Cyto-taxonomic relations", an attempt is made, first, to point out five cytological principles which provide the philosophical foundation for cyto-taxonomic research; second, to summarize some of the background of literature which serves for the establishment of these principles; and third, to review a few papers reporting outstanding cyto-taxonomic investigations which demonstrate the applicability of the method. Previous cytological work on *Medicago* and *Melilotus* is then outlined. Details of observations concerning somatic chromosome morphology and numbers are recorded for 23 species and five varieties of two additional species of *Medicago*, for four species of *Melilotus* and for three species of *Trigonella*. A summarized statement of all cytological observations is given.

In the discussion the following topics are considered in the light of the cytological data secured: general morphology of the somatic chromosomes and chromosome numbers found in the species studied, chromosome numbers and the annual or perennial habit in *Medicago*, different races in *Medicago falcata*, hybrid origin of *Medicago media*, taxonomic relations of *Medicago lupulina*, *Medicago carstiensis*, *Medicago obscura*, *Medicago rigidula* and *Medicago arabica*, and varieties of *Medicago hispida*. A few points of purely cytological interest are also discussed, viz., tetraploid chimeral areas, nucleolar remnants, and the peculiar anaphase chromosomes in somatic divisions.

In general it is concluded that these cytological studies have yielded information which can be utilized very effectively in clearing up some of the taxonomic problems, particularly, in the genus *Medicago*. It is predicted that the cytological information will also be useful to plant breeders who may contemplate interspecific hybridization among any of the species studied.

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Introduction

A large part of the historical development of the biological sciences has had to do with the classification of plants and animals into related groups, based on morphological similarities and differences and on ecological relations. But classifications based on these features have not always been satisfactory in establishing the true relationships among the different forms that make up tribes, genera or other taxonomic groups. Hence, in recent years cytological studies have come to be utilized to furnish new evidence of a fundamental character in the determination of taxonomic relationships. In many instances cytological evidence has confirmed original groupings of species or has aided in clearing up certain abstruse situations. The taxonomic relations involving the genus *Medicago*, when based on morphological features only, have not been satisfactorily established, neither in regard to interspecific relations within the genus nor in regard to the allocation of certain forms to this genus or to one of the similar genera *Melilotus* and *Trigonella*. It was thought that cytological evidence might throw some light on the true relations here as it appears to have done elsewhere.

In the genus *Medicago* the origins of certain species, varieties and supposed natural hybrids have been much debated and there seems to be some reason to believe that cytological studies might assist in determining the probable origins of these forms.

Moreover, the three genera mentioned above, particularly *Medicago*, include several species and varieties of great economic importance and, in order that improvements might be pursued intelligently by plant breeding methods, it is desirable that genetic relationships be correctly understood. Cytological studies may therefore be expected to assist indirectly in plant breeding procedure with these important crop plants.

In the cyto-taxonomic work here discussed emphasis has been given the genus *Medicago* while the two related genera, *Melilotus* and *Trigonella*, have been much less extensively dealt with and only for purposes of general comparison of their cytological features with those of *Medicago*.

Taxonomy of Medicago and Related Genera

The three genera under consideration have been placed by most taxonomists with *Ononis*, *Trifolium* and *Parochetus* in the tribe *Trifolieæ* of the *Leguminosæ*. Species of *Trifolieæ* are characterized chiefly by the trifoliate feature of the leaves, the leaflets of which are toothed on the distal portions of the edges. Entire leaflets occur rarely. Ten stamens are present, all of which are seldom united. Usually nine are united and one is free.

Different botanists have grouped the genera of trifoliates somewhat differently. Hegi (15) says that *Trifolieæ* are mostly divided into two subtribes *Oninidinæ* and *Trifoliinæ*, the first including the single genus *Ononis* while

the remaining five genera are grouped in the subtribe *Trifoliinæ*. O. F. Schultz as reported by Hegi (15) on the basis of seed characters divides the trifoliates into two tribes, viz., the *Trifolieæ* including *Ononis*, *Trifolium* and *Parochetus;* and *Trigonelleæ* including *Trigonella*, *Melilotus* and *Medicago*.

The demarcation of the genera *Trigonella*, *Trifolium*, and *Medicago* has been differently taken by different botanists. Hegi (15) reports Linnaeus, for instance, as placing *Melilotus* as a subgenus of *Trifolium*. Trautvetter (55) places it in *Medicago*. Certain species have been placed by some authors in *Trigonella*, and by others in *Melilotus*. As one instance, *Trigonella coerulea* has been placed in *Melilotus* by some botanists and in *Trigonella* by others.

The genera *Trigonella* and *Melilotus* appear to be more closely related to each other than either is to *Medicago*, on account of the jointed germ leaves which they possess in contrast to the more highly differentiated genus *Medicago*, in which the germ leaves are not jointed. On the other hand *Trigonella* and *Medicago* are similar in that the species of both are characterized by the presence of calcium oxalate crystals in the bracts. Hegi thinks one of the subgenera of *Trigonella*, which he terms *Eutrigonella*, is undoubtedly the original group relative (Sippi) of the *Trifoliineæ*. In his opinion this subgenus shows relations to *Melilotus* as well as to *Medicago* and *Trifolium*.

General characters of Medicago

The genus *Medicago* contains both annual and perennial species, and mostly herbaceous forms though there is one species which is a shrub, viz., *M. arborea* and a few which are inclined to be shrubby. Many species especially the annual species are prostrate in their growth habit; some of the perennial species are erect while other species are more or less intermediate. The leaves are alternate and trifoliate with stipules more or less attached to the petiole. The terminal leaflet is longer-stalked than the lateral leaflets. All the leaflets are more or less wedge-shaped and toothed on the distal edge. The flowers are mostly clustered in heads or short racemes, though sometimes they are single, arising from the axils of leaves. They are zygomorphic and the corolla is yellow, purple or variegated with colors somewhat intermediate between yellow and purple. The calyx has five nearly equal lobes which are sometimes mere tooth-like points. The pods are spirally coiled or sickle-shaped and seldom straight. The coiled pods are frequently beset with spines varying in size in different species. The seeds vary from one as in *M. lupulina* to many in a pod and are hard-coated. Germ leaves are never jointed in the petiole.

Medicago contains about 50 species which are distributed mainly in the Mediterranean areas, extending into central Europe, central Asia, and southwestern Asia as well as throughout northern Africa. Within the past century several of the more economically important species have been extensively introduced into North America, South America, Australia, New Zealand and other parts of the world.

General characters of Melilotus

The species of *Melilotus* are annual or biennial mostly smooth or sparsely hairy herbs, seldom semi-shrubby. Many possess a strong coumarin odor. The leaves are always trifoliate with long or oblong toothed leaflets. The terminal leaflet is stalked while the lateral leaflets are sessile. The stipules are joined to the petioles. The inflorescences occur in the axils of leaves as racemes, often very long and many-flowered. The flowers are mostly small and more or less drooping. The calyx is short bell-shaped with fine unequal lobe points. The corolla is monochromatic yellow or pure white except in *M. bicolor*, Boiss. et Bal. where it is flecked with blue. The pods are globular or ovate, mostly pointed. The seed is ovate single or two (seldom four) in the pod.

Melilotus contains about 20 species distributed in the subtropic zones of the old world. Some species have been widely introduced into the new world.

General characters of Trigonella

The species of *Trigonella* are annual, biennial and seldom perennial, and are small- or medium-sized herbs. Most species have a strong aromatic odor. The leaves are trifoliate and the leaflets are toothed. The terminal leaflet is distinctly stalked while the lateral leaflets are sessile. The stipules are often united far up with the petiole. The flowers are mostly small, in heads or short clusters (seldom single or paired) in the axils of leaves. The calyx is short, bell-shaped to tubular with five nearly equal lobe points. The corolla is yellow or blue. The pod is lineal or lanceolate and seldom ovate, and always many times longer than the calyx. It is straight or more or less curved, never coiled and often has a long beak. The seeds are usually several in the pod.

Trigonella contains about 70 species distributed chiefly in the eastern Mediterranean region extending into central Europe. Several species occur in northern Africa, one in South Africa and one in Australia.

Taxonomic grouping of species in Medicago

De Candolle (11) was one of the first botanists to attempt a grouping of species of *Medicago*. He based his classification almost entirely on characters of the pod, arranging the species into three sections, the third section of which he divided into three subsections. An outline of De Candolle's classification is as follows:

Section I. *Hymenocarpos*—Pods submembranous, compressed and curved; leaves 3-to-5-foliate; two species included *M. circinata* and *M. radiata*.

Section II. *Lupularia*—Pods kidney-shaped, curved or subsnail-like, glabrous or pubescent, margin entire; leaves 3-foliate. Sixteen species are listed including *M. lupulina*, *M. falcata*, *M. sativa*, *M. media*, *M. arborea*, etc.

Section III. *Spirocarpos*—Pods globular snail-like, pilose or glabrous, margin entire, wrinkled or spiny; leaves 3-foliate.

Subsection 1. Pods with thin margins and naked. Eight species are listed including *M. obscura*, *M. orbicularis*, *M. rugosa*, etc.

Subsection 2. Pods with thick margins and naked. Four species are listed including *M. tornata*, *M. tuberculata*, etc.

Subsection 3. Pods spiny. Forty-five species are listed including *M. coronata*, *M. littoralis*, *M. intertexta*, *M. carstiensis*, *M. rigidula*, *M. ciliaris*, etc.

A more recent classification of *Medicago* is that of Taubert (49) given in "Die Naturlichen Pflanzenfamilien", by Engler and Prantl. This classification is likewise based on pod characters, certain seed features also being used. The genus is here arranged in four sections, the fourth section of which is subdivided into seven subsections. Taubert's classification may be outlined briefly as follows:

Section I. *Hymenocarpoides*—Pods broad kidney-shaped or oval, flatly compressed, mostly with eight seeds, one side nerve missing. Only one species—*M. radiata*.

Section II. *Lupularia*—Pods kidney-shaped, mostly one-seeded, one side nerve missing. Two species, *M. lupulina and M. secundiflora*.

Section III. *Falcago*—Pods straight, sickle-shaped or rolled with open centres, one to many seeds, one side nerve missing. *M. arborea* the only shrub of the genus and a number of half shrubby or perennial species as *M. hybrida*, *M. cretacea*, *M. rupestris*, *M. prostrata*, *M. falcata*, *M. sativa*, *M. minima* and *M. media*.

Section IV. *Spirocarpos*—Pods spirally rolled with closed centres and many seeds.

A. One side nerve missing, nerves extending from the ventral suture run into the dorsal nerve or directly into spines.

Subsection 1. *Orbiculares*—Seeds yellow, reddish or brown, never black, radicle half as long as the seed or longer. Two species—*M. orbicularis* and *M. carstiensis*.

Subsection 2. *Intertextae*—Seeds black, radicle half as long or shorter than half the seed—*M. intertexta*, *M. echinus*, *M. ciliaris*.

Subsection 3. *Scutellatae*—Seeds never black, radicle shorter than half the seed—*M. scutellata* and *M. rugosa*.

B. Nerves of pod extending out from the ventral suture run into one of the side nerves parallel to the dorsal suture.

Subsection 4. *Rotatae*—Coils closely adjacent, separating walls between seeds missing or very low—*M. rotata*.

Subsection 5. *Pachyspirae*—Coils closely adjacent, seeds separated by thick and high separation walls, pod surfaces radially veined or somewhat veined along the side nerves—*M. obscura*, *M. littoralis*, *M. tuberculata*.

Subsection 6. *Euspirocarpae*—Coils loosely adjacent, seeds separated by partition walls—*M. arabica*, *M. hispida*.

Subsection 7. *Leptospirae*—Coils loosely adjacent, seeds not separated by partition walls—*M. laciniata*, *M. disciformis*.

Hegi (15) has also published a systematic classification of *Medicago* in his "Illustrierte Flora von Mittel Europa", which is worked out on a somewhat different basis. His classification tends in part to group the species somewhat similarly to that of Taubert, though in certain respects there appears to be some disagreement. His classification is limited since he includes only species found in Europe. He uses a dichotomous system dividing the genus into two subgenera, viz., *Eumedicago* and *Cymatium*. These are divided and subdivided forming a tree-like representation which may be regarded as having about six main branches. The species terminating the subdivision of each of these branches are presumably to be regarded as rather closely related. The six branches may be roughly described as follows:

Genus *Medicago*.

Subgenus *Eumedicago*—Pods more or less coiled or straight, seldom with spines; when spines are present coils are mostly over 1 cm. broad.

A. Pods kidney- or short sickle-shaped, single seeded; flowers small, yellow in short cluster—*M. lupulina*.

B. Pods spiral, sickle-shaped or nearly straight but then many-seeded, perennial species, flowers six to ten mm. long, seeds never black—*M. arborea*, *M. carstiensis*, *M. sativa*, *M. falcata*, *M. prostrata*.

C. Pods spiral, sickle-shaped or nearly straight but then many-seeded, annual species, flowers mostly smaller, always yellow—*M. radiata*, *M. orbicularis*, *M. rugosa*, *M. scutellata*, *M. intertexta*, *M. ciliaris*.

Subgenus *Cymatium*—Pods always dense snail-like, mostly under 1 cm. broad, spines mostly present, radial nerves run into lateral vein.

D. Coils of pod thick, closely packed, spines often short or entirely absent, seeds separated by strong partitions—*M. rigidula*, *M. aculeata*, *M. globosa*, *M. littoralis*, *M. obscura*, *M. tuberculata*, *M. murex*.

E. Coils thinner and looser, and always left hand turned, spines mostly long, smooth or weakly hairy plants, seeds separated by partition walls—*M. arabica*, *M. hispida*, *M. praecox*.

F. Coils thinner and looser, and always left hand turned, spines mostly long, silky hairy plants, seeds not separated by partition walls—*M. laciniata*, *M. minima*, *M. disciformis*.

The two above classifications may be compared by the following schematic arrangement:—

Taubert (49)		Hegi (15)	
Lupularia	*M. lupulina*	*M. lupulina*	Group A
Falcago	*M. arborea*	*M. arborea*	Group B
	M. sativa	*M. sativa*	
	M. falcata	*M. falcata*	
	M. prostrata	*M. prostrata*	
	M. hybrida	*M. hybrida*	
		M. carstiensis	
Hymenocarpoides			
	M. radiata		
Spirocarpos		*M. radiata*	Group C
Orbiculares.	*M. carstiensis*		
	M. orbicularis	*M. orbicularis*	
Scutellatae			
	M. rugosa	*M. rugosa*	
	M. scutellata	*M. scutellata*	
Intertextae			
	M. intertexta	*M. intertexta*	
	M. ciliaris	*M. ciliaris*	
Rotatae			
	M. rotata		
Pachyspirae			
	M. rigidula	*M. rigidula*	Group D
	M. littoralis	*M. littoralis*	
	M. obscura	*M. obscura*	
	M. tuberculata	*M. tuberculata*	
	M. murex	*M. murex*	
		M. aculeata	
		M. globosa	
		M. truncatula	
Euspirocarpae			
	M. arabica	*M. arabica*	Group E
	M. hispida	*M. hispida*	
		M. praecox	
Leptospirae			
	M. laciniata	*M. laciniata*	Group F
	M. minima	*M. minima*	
	M. disciformis	*M. disciformis*	
	M. Aschersonia	*M. Schimperiana*	

The associations as arranged by Taubert and Hegi are thus seen to agree for the most part; however, there are evidently some discrepancies. *M. radiata* is placed by Taubert in a group by itself while Hegi associates it with *M. orbicularis*, *M. rugosa* and *M. scutellata*, though rather remotely. Taubert associates *M. carstiensis* with *M. orbicularis* while Hegi places it with *M. arborea*, *M. sativa*, *M. falcata*, *M. hybrida* and *M. prostrata*.

Taxonomy of Medicago sativa and Medicago falcata

There has been some disagreement among botanists in regard to these two species. Hegi (15) for instance, appears to have included in one species all forms usually included in these two species, as well as intermediate forms. Oakley and Garver (34) discuss the botanical history of *Medicago falcata*

especially and incidentally refer also to *Medicago sativa* and to hybrids between these two species as well. Apparently early botanists confused these species and their hybrids, and different writers used different names for similar forms or, because of the great range of forms, the same name was frequently attached to different morphological types. Oakley and Garver also point out that even recent writers differ somewhat in regard to the taxonomic relation of these two species. Some regard *M. falcata* as a variety or subspecies of *M. sativa*. Others again are of the opinion that *M. falcata* is the true species and that *M. sativa* is only a subspecies or a cultivated variety of it. Linnaeus at one time suggested the latter arrangement.

Medicago falcata has been studied extensively by Oakley and Garver (34) especially with regard to the great variability of morphological features to be found within the generally accepted limits of the species. These authors list 22 botanical names which have been applied to species and varieties whose origin and relationship to *Medicago falcata* are not clear. After studying all the forms available to them, they decided that a botanical classification on the basis of several characters, for example, color, size, and abundance of flowers, type of pod and seed habit, foliage characteristics, stem types and root systems would not be practical. They therefore grouped the forms based on plant type with regard to erectness, type of crown, and general habit of growth. The groups varied from low-growing prostrate forms through decumbent, ascending to suberect forms, with more or less continuous intergradations. Oakley and Garver thus emphasize the extreme variability of form in the species *M. falcata*.

Taxonomic relations of Medicago media

There are many intermediate forms between *M. sativa* and *M. falcata*, which include the famous variety, "Grimm", and several other varieties of very great economic importance. These forms are vigorous in growth, have the so-called variegated flowers and smooth coiled pods, are adaptable to a wider range of climatic conditions than *M. sativa*, and they are more productive of seed than *M. falcata*. Although the origin of these forms has been much debated there seems to be little doubt that they arose by natural hybridization between *M. sativa* and *M. falcata*. They are in general intermediate in morphological characters between these two species and forms similar to them have been produced through artificial hybridization of the two species. Moreover the two species are known to be reciprocally cross fertile under natural conditions producing fertile hybrids in both cases. Waldron (56) has recommended the mixed planting of *M. sativa* and *M. falcata* designedly to obtain natural hybrids for economic purposes.

F. Hy (23) has studied some of the forms intermediate between *Medicago sativa* and *Medicago falcata* as found in the valley of the Loire. He cites the opinions of different botanists regarding *Medicago media* as originally described by Persoon. Some of these botanists regarded it as of hybrid origin; others considered it as a type subordinate to *M. falcata* while others thought it to be a poorly defined form of *M. falcata*. Hy found three types to which

he gave the names *Medicago cyclocarpa* (generally regarded as *M. media*), *Medicago spuria*, (hybrid from *M. cyclocarpa* × *M. sativa*) and *Medicago lilacea* (probably another hybrid segregate from *M. cyclocarpa* × *M. sativa*). He regarded his *M. cyclocarpa* as non-hybrid in origin chiefly because of its high fertility. *M. spuria* has yellow flowers and is practically sterile while *M. lilacea* has purple flowers with yellow admixture and is nearly sterile.

It would seem, however, that Hy's conclusions ought to be appraised rather cautiously since he apparently confined his observations to a comparatively small amount of material from a rather restricted area, and also since it is now known that entirely fertile hybrids are found frequently to be obtainable from inter-crossing certain distinct species of other plant families.

Varieties in many species of Medicago

McKee and Ricker (28) point out that *Medicago* as commonly accepted by botanists includes about seven perennial species with about 16 subspecies and about 37 annual species with about 80 subspecies. The large number of subspecies in this genus would suggest a high degree of variability involving small morphological differences. And since species in some instances are differentiated by rather slight morphological differences it might be suspected that the accepted classifications may contain some discrepancies which cytological studies might help to adjust. It also seemed as if it might be interesting to determine cytological differences, if any, between the subspecies or varieties of species. In this connection the varieties of *Medicago arabica* and *Medicago hispida* are of interest since these species are very common in California and other southwestern States. *M. arabica* (L) is characterized by spiny pods while its subspecies *M. arabica inermis*, Ricker, is similar in all respects except that it has pods without spines. *Medicago hispida* has at least six subspecies, viz., *M. hispida denticulata*, *M. hispida confinis*, *M. hispida reticulata*, *M. hispida apiculata*, *M. hispida nigra* and *M. hispida terebellum*. These all differ slightly, particularly in characters of the pod. *M. hispida denticulata* has pods with long spines, *M. hispida apiculata* has pods with short spines while *M. hispida confinis* has pods without spines.

In this rather lengthy treatment of the taxonomy of *Medicago* an effort has been made to emphasize the fact that the taxonomy of the genus based on morphological characters is unsatisfactory in some parts. In commenting on this point Scofield (43) stated after studying material collected by himself in North Africa, "In attempting to identify the various Algerian species, the literature of the group has been examined, and as a result it seems certain that a complete revision will be necessary before these species can be correctly identified and their relationships properly understood".

Classification of Melilotus

Taubert's (49) grouping of the species may be briefly outlined as follows:

Section I. *Lopholobus*, Boiss—Pods spongy-papery, short, with 8-10 somewhat undulating longitudinal ribs. Single species, *M. bicolor*, Boiss et Bal. in Phrygia with white flowers. Standard and top of other petals blue violet.

Section II. *Campylorytes* D.C. (Gyrorytes Koch)—Pod concentric wrinkle-folded. Four species of which *M. messanensis* Desf. and *M. sulcata* Desf. are distributed in the Mediterranean region and the Canary Islands.

Section III. *Plagiorytis* Ser.—Pod with light curved wrinkles, three species of which *M. officinalis* Desr. is best known. It occurs in all Europe, south-western Asia and in Siberia.

Section IV. *Coelorytis* Ser.—Pod is wrinkle-veined. About 12 species.

A. Biennial plants—*M. alba* Desr., *M. dentata* Pers., *M. altissimus* Thuill.

B. Annual plants—*M. italica* Desr., *M. indica* All., *M. ruthenica* M.B.

Classification of Trigonella.

Taubert's (49) grouping of the species of *Trigonella* may be briefly outlined as follows:

Section I. *Eutrigonella*—Pods linear, lance-like or oblong, straight, curved or hooked, cylindrical or more or less compressed, but never flat and paper-like.

A. Pods upright.

Subsection 1. *Capitatae*—Flowers in dense mostly long-stalked heads, blue.

Subsection 2. *Gladiatae*—Flowers single or paired in axils, whitish yellow or violet.

Subsection 3. *Bucerates*—Flowers umbels or short racemes, always yellow.

B. Pods curved backwards.

(a) Pods constricted between seeds.

Subsection 4. *Isthmocarpae*—Pods slender cylindrical, short beaked.

Subsection 5. *Cylindricae*—Pods cylindrical, gradually becoming more slender towards the point, non-beaked.

(b) Pods not constricted between seeds.

I. Pods scarcely beaked, not hooked.

(1) Pods cylindrical with indefinite suture lines.

Subsection 6. *Falcatulae*—Pods linear, seldom oblong egg-shaped, more or less compressed with strong suture lines.

II. Pods distinctly beaked or hooked.

Subsection 7. *Callicerates*—Pods umbel, columnar, long beaked.

Subsection 8. *Uncinatae*—Pods dense head forming, short compressed, hooked.

Section II. *Pocockia*—Pods flat, paper-like with parallel anastomosing cross nerves.

A. Pods umbel, half-moon-shaped with unwinged sutures.

Subsection 1. *Lunatae.*

B. Pods short or very short racemes, keeled on the edge, toothed or winged.

Subsection 2. *Ellipticae*—Pods elliptical or lance-like, keeled along the upper suture.

Subsection 3. *Pedenatae*—Pods curved, ciliated-toothed on both sutures.

Subsection 4. *Samaroideae*—Pods egg-shaped, winged all round.

Geographical distribution

The species of Medicago are indigenous to the subtropical and temperate regions of the northern hemisphere of the Old World. The annual and tender species are confined to the Mediterranean region (southern Europe and northern Africa) and western and central Asia. The perennial and more hardy species are more widely distributed particularly towards the north. *Medicago falcata* extends to the 69th parallel of latitude in northern Europe and to the 63rd parallel in Siberia. It extends east to Pekin and south to northern India, Persia and Syria but not into northern Africa. *Medicago sativa* has a similar but much more restricted distribution towards the north and east. *Medicago lupulina* has a distribution similar to that of *M. falcata*.

A recognition of the great economic value of certain species especially *Medicago sativa* (alfalfa), was responsible for their introduction and distribution into South America and Mexico in the 18th century and into southwestern United States about the middle of the 19th century. *Medicago sativa* or similar perennial forms were introduced into eastern United States also in the 19th century and from the east and west, alfalfa, as all these forms are now called, has spread almost entirely over the United States and Canada. It has been introduced also into Australia, New Zealand and South Africa in recent years. Many annual species have been sought out in their native habitats in Europe, Asia and north Africa and have been exploited for agricultural purposes in the United States with the result that certain species are now rather widely distributed and are growing wild; for example, *Medicago arabica* is scattered through many of the southern states while *Medicago hispida* and some of its subspecies are distributed throughout the southwestern states especially in California. *Medicago lupulina* has also gradually spread to practically all parts of United States and Canada where moisture conditions are favorable.

The species of *Melilotus* and *Trigonella* were originally distributed similarly to those of *Medicago*, *Melilotus* extending northerly and *Trigonella* more southerly. Like *Medicago*, *Melilotus* and *Trigonella* have been introduced into North America and certain species of *Melilotus* in particular have attained very general distribution over the settled portions of this continent.

Grouping of species studied

The species and subspecies examined for purposes of the cytological study herein reported, have been arranged according to Taubert's (49) grouping as follows:

Genus *Medicago*

Section II.	*Lupularia*	*M. lupulina*
Section III.	*Falcago*	*M. sativa*
		M. falcata
		M. media (Grimm)
		M. media
		M. platycarpa
		M. ruthenica

Section IV. *Spirocarpos*

Subsection 1.	*Orbiculares*	*M. orbicularis*
		M. carstiensis
		M. soleirolii
Subsection 2.	*Intertextae*	*M. intertexta*
		M. echinus
		M. ciliaris
Subsection 3.	*Scutellatae*	*M. scutellata*
		M. rugosa
Subsection 4.	*Rotatae*	*M. rotata*
Subsection 5.	*Pachyspirae*	*M. rigidula*
		M. littoralis
		M. tuberculata aculeata
		M. murex
		M. muricata
		M. obscura
Subsection 6.	*Euspirocarpae*	*M. arabica*
		M. hispida var. denticulata
		M. hispida var. confinis
		M. hispida var. terebellum
		M. hispida var. nigra
Subsection 7.	*Leptospirae*	*M. laciniata*
		M. coronata

Genus *Melilotus*

Section II.	*Campylorytis*	*M. sulcatus*
Section III.	*Plagiorytis*	*M. officinalis*
Section IV.	*Coelorytis*	*M. alba*
		M. indica

Genus *Trigonella*

Section I. *Eutrigonella*

Subsection 1.	*Capitatae*	*T. coerulea*
Subsection 2.	*Gladiatae*	*T. foenum graecum*

Section II. *Pocockia*

Subsection 4.	*Samaroideae*	*T. cretica*

Cyto-taxonomic Relations

Cytological Principles

Cytological studies in recent years have centred largely around the chromosomes, and as an outgrowth of extensive investigations certain cytological principles have geen generalized and rather well established, which have proved to be very helpful in taxonomic problems. The most important of these cytological principles are: (1) constancy of chromosome number in an individual or in a stable genetic group of individuals, e.g. a race or species; (2) constancy

of chromosome size and shape within an individual or within a stable group of individuals; (3) genetic continuity of the chromosome; (4) if any change in the number or morphological features of the chromosomes occurs the new condition tends to be reproduced in succeeding cells and generations; (5) similarity of chromosome features in two or more races or species within a relatively small group indicates close phylogenetic relationship, while diversity between chromosome complements indicates more remote relationship. The following brief discussions may serve to establish for present purposes these cytological principles.

Chromosome number

According to Sharp (44), Boveri, van Benedin and Strasburger in the later decades of the last century were among the first to note the constancy of chromosome number within a given species. Since that time numerous cytologists have investigated a great variety of plant material. A few of the outstanding workers with plant materials may be mentioned. Sakamura (39, 40), and Sax (41, 42), both working with *Triticum;* Tackholm (47, 48), on *Rosa;* Heilborn (16), on *Carex;* Clausen, J. (8), on *Viola;* Hollingshead and Babcock (21) on *Crepis;* and many others. Tischler (53, 54) and Winge (60) have collected data on chromosome numbers for numerous species and have discussed their general biological significance.

There have been a few who have disputed the principle of constancy of chromosome numbers, chief among whom are Fick and Della Valle according to Wilson (59), p. 833, and Sharp (44), p. 179, but the evidence overwhelmingly favors the principle, and it may therefore be considered as definitely established.

Constancy of size and shape of chromosomes

One of the most striking results of recent cytological investigation has been the discovery of distinctive size and shape features of chromosomes when observed at the somatic metaphase stage. Each parent provides a set of chromosomes to a new individual and each chromosome is then present in duplicate in each nucleus of the new individual. Given chromosome sets have often been found to contain one or more pairs which are distinguishable by size differentiation. Workers with *Datura* have found distinct size classification possible in this genus. Heilborn (16) found size distinctions in many species of *Carex.* Newton (33) found length differences in *Galtonia* and Taylor (51) found similar distinctions in *Cyrtanthus parviflorus.*

In addition to size characteristics, different shape features have been recognized, for example, V-shaped, J-shaped, rod-shaped chromosomes occur with constancy. Constrictions have been found to be located at definite positions on some chromosomes, usually related to the point of spindle-fibre attachment. Satellites of definite size and position are attached to certain chromosomes, and these occur with constant regularity. As in the case of size characteristics, these shape features are the same for both homologues of any given pair. Many cytologists have noted these morphological features of the somatic chromosome. Taylor (50) observed in *Gasteria* pronounced size

differences, constrictions and points of spindle-fibre attachments. He found these morphological features to be constant in root tips, perianth, general ovary tissue, anther walls and archesporial tissue. Taylor (51, 52) studied several other species in which he found constancy of relation between position of constrictions and of spindle-fibre attachment. He also found constancy of occurrence of satellites on certain chromosomes. He came to the conclusion that a series of chromosome features may be established which is characteristic of a genus, of a species or perhaps only a race or of an individual. Mann (26) studied 19 species of *Crepis* and found that in certain species chromosome individuality was very distinct, and could be used as a diagnostic character in specific determination. Navashin, M. (30) gave a complete description of satellites in 10 species of *Crepis* by which these species could be identified. Hollingshead and Babcock (21) found that the relative length of arms composing individual chromosomes in *Crepis* are in general constant. They also found the regular occurrence of satellites in all of the 67 species of *Crepis*. Ruttle (37, 38) studied chromosome morphology in certain forms of *Nicotiana* and found it possible to classify the chromosomes on the basis of constantly occurring morphological features of chromosome length, and presence or absence of satellites.

The works quoted here and many others have thus definitely established the existence of specific morphological features of chromosomes and their constancy in normal individuals and in stable genetic groups of individuals.

Genetic continuity of chromosomes

This principle is strongly supported by the previously mentioned principles, viz., the constancy of chromosome number and the constancy of morphological features of chromosomes. In addition another line of evidence may be mentioned, viz., the persistence of visible chromosome limits during the interphase. In rapidly growing tissues it has frequently been observed that the telophase chromosomes do not completely disorganize into the reticulum of the interphase. Instead they only partially disintegrate, the boundaries of their ramifying branches all remaining intact, so as to maintain in a visible condition the entirety of each chromosome throughout the interphase. Thus the telophase chromosome may be followed through the interphase into the prophase chromosome without loss of its spacial limits. In certain animal material, the spermatogonia of *Phrynotettix*, for example, Wenrich (57) found that the telophase chromosome forms its own vesicle about it, and in many cases the limits of these vesicles do not entirely disappear during interphase. The prophase chromosome is formed from the chromatic material within the telophase vesicle.

Cases of persistence of chromosome limits through the interphase suggest that possibly the chromosomes universally retain their own individuality, though usually in an invisible condition, from telophase to prophase.

Tendency for altered conditions in chromosome number or in chromosome morphology to perpetuate themselves

There is much evidence that might be cited in support of this principle but it is unnecessary to deal extensively with this question here. Chromosome changes generally occur in somatic mitosis, in meiosis or in heterogamous syngamy.

In the first instance, viz. somatic mitosis, the change is usually a multiplication of the entire chromosome set. Once the number is multiplied the new polyploid condition tends to reproduce polyploid cells having the new chromosome number. Numerous examples of tetraploid areas in root tips might be cited. Blakeslee and Belling (5) found tetraploid shoots on diploid *Datura* plants. Navashin, M. (31) and Hollingshead (19) found polyploid areas in root tips in certain species of *Crepis*. Hollingshead (20) and Babcock and Navashin (3) found numerous diploid areas in root tips of haploid plants of *Crepis capillaris*. Ruttle (38) found diploid chimeras and diploid roots formed from haploid plants of *Nicotiana tabacum purpurea*. Changes in somatic mitosis may be due to non-disjunction, in which case a cell with a new number, $2n+1$ or $2n-1$, may arise. From such a cell a trisomic or a monosomic shoot may develop. Blakeslee and Belling (5) found chimeral branches on *Datura* plants some of which had $2n-1$ chromosomes and some of which had $2n+1$ chromosomes. Evidently each of these chimeras originated through non-disjunction in some meristematic cell of the young plant and the new number of chromosomes was perpetuated in all cells genetically related to the original cell in which the non-disjunction occurred.

In the second instance, viz. chromosome changes during meiosis, gametes may arise with irregular chromosome numbers or morphological complexes. Diploid gametes occur through duplication in meiosis, gametes with varying chromosome numbers may arise from meiotic non-conjunction or meiotic non-disjunction, or fragmentation of lagging univalent chromosomes may occur, in which case the gametic set may be altered to the extent of containing a chromosome fragment instead of the entire chromosome. These phenomena provide means by which normal chromosome numbers or chromosome associations may be changed to give gametes having numerous variations from the normals. These chromosome variations in the abnormal gametes tend to perpetuate themselves in zygotes which they help to initiate through syngamy, if the gametes are not so abnormal as to be non-functional.

In the third instance, viz. changes in heterogamous syngamy, gametes with different chromosome complexes, if they are sufficiently compatible, form zygotes with chromosome complements different from that of either of the parental forms. This results from interspecific hybridization between species whose chromosome complements are distinctly different in number or morphological characteristics. Moenkhaus (29) was able to distinguish between the long chromosomes of *Fundulus* and the short chromosomes of *Menidia* in the hybrids obtained by crossing these two forms. Mann (26) studying hybrids, *Crepis setosa* × *C. tectorum* and *C. setosa* × *C. dioscoridis* was able to

identify the chromosomes of *C. tectorum* and *C. dioscoridis* by size characteristics, when they were associated with *C. setosa* chromosomes in the hydrid complements. Babcock and Collins (1), Collins and Mann (10), Navashin (32) and Collins, Hollingshead and Avery (9) found individual parental chromosomes in interspecific hybrids in *Crepis*. Chromosomes introduced into a zygote appear to retain their identity whether syngamy is homogamous or heterogamous.

The tendency to chromosome stability promotes the general permanency of specific forms, while the rare changes in chromosome number or in chromosome morphology appear likely to be associated with specific changes. The fewer the permanent chromosome changes, presumably, the fewer are the permanent species or race differentiations.

Similarity of chromosome features of two or more races or species, within a comparatively small group, indicates close phylogenetic relationship, while diversity of chromosome complements indicates more remote relationship

This principle is based on general taxonomic and cytologic knowledge, and is supported by some rather convincing cyto-taxonomic investigations. It cannot be interpreted as indicating that species which are widely different morphologically must necessarily be closely related if their chromosome complements are visibly indistinguisable. The principle at present would seem to find its greatest value in supplementing morphological, genetical and ecological evidence of kinship. Heilborn (16) concluded that in *Carex* nearly related species possess the same or adjacent chromosome numbers. He also points out that groupings of adjacent chromosome numbers are obtained when the species are arranged according to systematic relationship. Sharp (44) in discussing the phylogenetic significance of chromosomes says: "A certain amount of speculation regarding the general phylogenetic significance of chromosome number has been indulged in, but students of the subject have reached the conclusion that the data at hand do not warrant many broad generalizations. So far as the large groups are concerned, there appears to be no correlation between taxonomic position and structural complexity on the one hand, and the number, length, width, or volume of the chromosomes on the other. But it is generally agreed that within restricted circles of affinity the number of chromosomes, as well as their size and form, may often afford important evidence in the determination of genetic relationship". Babcock and Navashin, M. (3), in discussing chromosome morphology as a specific character in *Crepis*, remark: "It may be stated that, as a rule, closely related species have similar chromosomes, while less closely related forms have chromosomes differing more or less widely. However, a few exceptions to this rule are known". This remark sounds a word of caution against placing too much reliance in the universal application of this principle.

Review of pertinent cyto-taxonomic literature

An attempt will here be made to review only a few of the more outstanding and recent papers involving cyto-taxonomic relationships with reference chiefly to plant material. Most of these may be regarded as demonstrations of cytological methods utilized for taxonomic purposes.

McClung (27) discusses the general topic of the relation between cytology and taxonomy, and by means of concrete examples advances grounds for his theoretical belief in a parallelism between germ cell structure and taxonomic relations. He attempts to explain the causal connections between the two sets of phenomena in the light of present knowledge. The cytology and taxonomy of *Acrididae* and *Locustidae*, two families of *Orthoptera* are discussed. McClung found a multiple chromosome, a hexad in the first spermatocyte in all species of the genus *Hesperotettix* of the family *Acrodidae* and it exhibited variations or modifications of size and proportion in the different species of this genus. The differences between this chromosome in the three forms of *Hesperotettix* were considered just as specific as any that might have been chosen from ordinary somatic characters.

Robertson (36) worked on the relationships shown in chromosomes of *Tettigidae* and *Acrididae*. He adheres to the general hypothesis of McClung in regard to the relationship between cytology and taxonomy and states that as the number of species of *Orthoptera* examined for chromosome conditions increases from year to year the results tend to confirm the correctness of McClung's view. The author says: "In the chromosomes of *Orthoptera* we seem to be dealing with morphological structures possessing a definite relative size and constitution as permanent as the cell itself. Their size relations and their behavior are probably constant, not only for all cells of an individual, but every individual of a species. These size relations may vary, it is true, to a slight extent in the different species of a genus, as would be expected, and still more when the different species of a genus are compared; but they may be reasonably constant within these limits for the subfamily, and in many cases for the family. Thus the degree of relationship is expressed as accurately in the nucleus as in any of the external body characters".

One of the most outstanding works in which cyto-taxonomic relations were extensively studied is by Heilborn (16, 18) on the genus *Carex*. Fifty-six species and two hybrids in all were studied in which 24 different chromosome numbers were found ranging from 9 to 56. These numbers could not be arranged in multiple series and hence appeared to be an exception to the rule. Heilborn studied the size of the chromosomes and thought that in general the size decreased with the number. Considerable size and shape variability was found within individual chromosomes. By exact measurements of chromosomes in several species he was able to classify the chromosomes in each species into "long", "medium", and "short" groups. In *Carex pilulifera* and *C. panecia* Heilborn thought a long chromosome equalled a medium one plus a short one in length, and this suggested that the latter two had at some time originated as a result of transverse division of a long chromosome.

Heilborn was particularly interested in the possible relationship between the chromosome numbers and the taxonomy of the genus, and from his results he concludes that there is fairly good proof of the validity in this genus of the relation between chromosome numbers and the systematic position of the species. He also expresses the belief that chromosome numbers must be of great importance in all attempts to trace the phylogeny of the genus *Carex*.

Heilborn (17) reports chromosome studies on *Draba*. This work was done to test the limits of the species and their uniformity with the aid of chromosome numbers. The numbers were found to form a series, viz. 8, 16, 24, 32 and 40. Some previous taxonomic difficulties between *Draba rupestris* and *D. magellanica* were removed. *D. rupestris* had 24 chromosomes and was cytologically uniform, while *D. magellanica* was found to consist of three cytological types with 24, 32 and 40 as chromosome numbers. Heilborn thinks that, judging by cytological evidence, these two species ought not to be confused. He also thinks that in *D. magellanica* the three forms may be related in spite of their different chromosome numbers.

Babcock and Lesley (2) applied cytological methods to taxonomic studies in *Crepis*, partly with a view to testing the cyto-taxonomic method in a genus regarded as favorable for such research, especially as the genus has presented considerable difficulty to taxonomists. Chromosome number and size were studied. The numbers found were 3, 4, 5, 6, 8, 9 and 20 pairs. It was suggested that Hoffman's classification should be revised on a more natural basis taking into account the cytological data secured. It was found that species which stand apart in the classification also differ decidedly from the rest in size or number of chromosomes. Chromosome size was found to be a better criterion of relationship than number alone although closely related species usually had the same chromosome number. *Eucrepis*, one of the sections of Hoffman's classification was found to be too heterogeneous both taxonomically and cytologically to be retained as a section and consequently new groupings were considered to be needed. The authors were evidently satisfied with the cyto-taxonomic method. In regard to this and their suggested new grouping they say: "It is evident that generally speaking there is a definite correspondence between the taxonomic position of the species studied and their chromosome number, and especially with chromosome size, and that the new taxonomic grouping increases this correspondence".

Kihara and Ono (24) found a remarkable polyploid series of chromosome numbers for certain species of *Rumex*. In the section *Lapathum* subsection *Platypodium*, *R. bucephalophorus* was found to have eight pairs while in subsection *Eulapathum* of the same section, species were found with haploid numbers of 10, 20, 30, 40, 50, 60 and 100. The authors think that species with the missing numbers of this series may yet be found. In section *Acetosa* one species, *R. scutatus* was found to have 10 as the haploid number while *R. arifolius* and *R. nivalis* had 6+X and 6+Y+Y chromosomes in each. The polyploid series in subsection *Eulapathum* is particularly interesting in that it indicates the probable close relationship of species showing a polyploid series.

Longley (25) found that *Fragaria* species could be grouped into classes similar to those found in other polymorphic genera of the *Rosaceae* according to the numbers of chromosomes found at diakinesis. The species worked with could be grouped into three classes, viz.,

1. Diploid group with seven as the basic number.

2. Hexaploid group with 21 as the haploid number.

3. Octoploid group with 28 as the haploid number. The octoploid group includes two American species and most of the horticultural varieties.

Clausen, J. (8) studied chromosome numbers and the relationships of 30 species of *Viola*. The haploid numbers found by him and others were, 6, 7, 10, 11, 12, 13, 17, 18, 20, 24, 26, 27, 30 and 36. They fell more or less into definite groups forming a 6-series, a 12-series and a 10-series. *Viola* has five sections most important of which in point of numbers of species in the section are *Nominium* and *Melanium*. In the former there are 10 and 12-series; in the latter section a 10-series and a 6-series occur, the 6-series, however, having rather numerous aberrations. In one species, *Viola Kitaibeliana*, three chromosome numbers were found, viz., 7, about 12 and 18, all of Caucasian types. Clausen regards these three as a diploid, a tetraploid and a hexaploid respectively and mentions them as varieties. In general he found that species of the same subgroup belong as a rule to the same series of chromosome numbers. In the *Melanium* section a new subdivision was made on the basis of chromosome numbers, the section being now divided into two groups, *Calcaratae* (the 10-series) and *Tricolores* (the 6-series). The author states that chromosome numbers afford not only an aid to the grouping of species, but constitute an important factor determining whether two types are specifically distinct or not.

Sveshnikova (46) studied 30 species of *Vicia* in an attempt to relate karyological features with the external morphology and the taxonomy of the genus. Three main groups were represented—*Ervum* with 14 chromosomes in all species, *Cracca* with 12, 14, 24 and 28, and *Euvicia* with 12 and 14 chromosomes. For each species the somatic chromosomes were classified into four types according to the ratio shown by the lengths of the arms and the presence or absence of heads and satellites. Idiograms were made for 25 species having 12 and 14 chromosomes (there were also two species with 24 and one with 28 chromosomes) illustrating for each species the proportions of the different chromosome types. A classification key was prepared based almost entirely on the proportions of the different types of chromosomes in each species. The authoress then found that her classification key was in general agreement with Ascherson's classification which was based on external morphological characters, with the exception of the end of the key, where she singled out *Vicia narbonensis* into a separate section. This work appears to demonstrate a case of remarkable correlation between chromosome features and taxonomic relations within a genus.

Wexelsen (58) studied the number and morphology of chromosomes in *Trifolium* of the *Leguminosae* family. Ten American species had diploid numbers of 16, 32 and 48. No representatives of the seven series common in European species of *Trifolium* were found among the American species. The chromosomes were in general small but varied considerably in size. The author found no parallelism in the differentiation of the chromosome complexes and of the external morphology. Species taxonomically far removed and very different morphologically had very similar chromosome complexes. And on the other hand closely related species with different chromosome complexes were found. This he believes to be a case of parallel variation. The presence of the same particular chromosome features in several diverse species he thinks may be due to independent parallel mutations in chromosome features rather than to common chromosome descent. Wexelsen thinks it will not be easy on the basis of chromosome morphology to trace the mutational relationship and origin of species in *Trifolium*. He also points out that it is probably only in the narrowest taxonomic groups that there is a similarity in chromosome morphology which points to common descent and that it is probably here that the study of chromosomes may be of help to the taxonomist.

Hollingshead and Babcock (21) studied rather extensively the relation of chromosomes and phylogeny in the genus *Crepis*. Their recent investigations add 27 species to those previously studied, making 70 species, all of which they consider in their suggested phylogenetic system of classification. The somatic chromosome numbers reported are 6, 8, 10, 12, 14, 15, 16, 18 and 40± for Old World species and 14, 22, 33, 44, 55? and 88? for American species. A phylogenetic chart is presented for 67 species, and the phylogenetic relationships of the subgenera and species are discussed with special reference to morphological and cytological features. The general phylogenetic significance of chromosomes is discussed at some length, special emphasis being given the probable means by which evolutionary changes in chromosome numbers, size, shape, etc. may have been brought about in the evolution of *Crepis*. The authors review several current hypotheses as to means of chromosome changes, viz., elimination of a pair of chromosomes following irregular meiosis, fusion of non-homologous chromosomes, gradual diminution and disappearance of chromosomes, hybridization, polyploidy, transverse segmentation, gradual accumulation of minute changes in chromosomes, etc. Their studies on *Crepis* did not appear to supply evidence lending strong support to these hypotheses. The authors believe that evolutionary changes have occurred in *Crepis* involving: (a) decrease in chromosome number possibly brought about by gradual diminution of a particular chromosome pair or by interspecific hybridization; (b) increase in chromosome numbers brought about by doubling the chromosome number, hybridization, and amphidiploidy; (c) change in size probably occasioned by the gradual accumulation of small alterations in particular chromosomes, and (d) changes in shape possibly brought about by a shifting of the fibre-attachment constriction rather than from a lengthening of one arm and a shortening of the other as hypothesized by Heitz. The writers finally

emphasize their most important generalization from the point of view of phylogeny and classification, viz. that morphologically similar species have similar chromosomes.

SUMMARY

In this section, 'Cyto-taxonomic Relations', an attempt has been made, first, to point out five cytological principles which provide the philosophical foundation for cyto-taxonomic research; second, to summarize some of the background which serves for the establishment of these principles; and third, to review a few of the outstanding cyto-taxonomic investigations which demonstrate the applicability of the method.

Previous Cytological Studies on Medicago and Melilotus

Very little cytological work has been done on *Medicago* or on *Melilotus* and none at all appears to have been done on *Trigonella*.

Karpechenko (1925) according to Tischler (54) reported haploid chromosome numbers as follows: *Medicago lupulina*, 8; *M. platycarpa*, 8; *M. sativa*, 16 and *M. falcata*, 16.

Ghimpu (14) studied the chromosomes of 14 species of *Medicago*. Root tip material obtained from germinating seeds was used for a study of somatic chromosome number, shape and size. The species studied and their chromosome numbers found were as follows:

Section (of the genus)	*Species*	*2n*	*Section* (of the genus)	*Species*	*2n*
Falcago	*M. sativa*	32	*Spirocarpos*	*M. maculata*	16
Lupularia	*M. lupulina*	16	*Spirocarpos*	*M. spaerocarpa*	16
Spirocarpos	*M. scutellata*	32	*Spirocarpos*	*M. rigidula*	16
Spirocarpos	*M. orbicularis*	16	*Spirocarpos*	*M. tornata*	16
Spirocarpos	*M. echinus*	16	*Spirocarpos*	*M. fenoreana*	16
Spirocarpos	*M. disciformis*	16	*Spirocarpos*	*M. truncatula*	16
Spirocarpos	*M. minima*	16	*Spirocarpos*	*M. helix*	16

In the same species the author found that differences were indeterminable in respect to shape of chromosomes, but variations from species to species were large enough to be detected. Four groups of species were suggested based on number and size of chromosome, viz.:

species with 16 large chromosomes, e.g., *M. orbicularis*

species with 16 medium chromosomes, e.g., *M. echinus*

species with 32 medium chromosomes, e.g., *M. sativa*

species with 16 small chromosomes.

The author claims that this grouping does not correspond with that established by taxonomists according to characters of the pod.

Castetter (6) studied cytologically the annual form of *Melilotus alba* and found eight pairs of chromosomes in meiosis and eight chromosomes in each microspore. He also found two kinds of pollen grains, one kind normal and the other kind three or four times as large, and he thought the occurrence of annual and biennial varieties was associated with these two kinds of pollen grains. The same author (7) found that in both the annual and the biennial varieties of *M. alba* the haploid number of chromosomes was eight and the diploid number was 16. He does not appear to have made any study of chromosome morphology.

Elders (13) studied somatic chromosomes in root tips of *Melilotus alba*, *Melilotus alba annua*, *Melilotus officinalis* and *Medicago sativa*. All three forms of *Melilotus* had 16 while *Medicago sativa* had 32 as the somatic chromosome numbers. No data is given on chromosome morphology, but the author's figures indicate similarity of size and shape in all four forms except that for *Melilotus alba* the chromosomes are figured as somewhat thicker than they are for the other species.

Materials and Methods

The materials used in this study were grown from seeds furnished by the late Professor P. B. Kennedy and Professor B. A. Madson of the University of California, Mr. Roland McKee of the Bureau of Plant Industry of the United States Department of Agriculture, and by European and other Botanical Gardens through Professor T. H. Goodspeed of the University of California. Wild-growing plants of *Medicago hispida denticulata*, *M. hispida confinis* and *Melilotus indica* were utilized while seeds of *Medicago media* ("Grimm" alfalfa), of *Melilotus alba* and of *Melilotus officinalis* were obtained from the University of Alberta, Canada.

The seeds were germinated in sterilized soil and the seedlings transplanted individually into small pots. Root tips were taken later and fixed in Karpechenko's solutions for 24 hr. In a few cases the root tips were obtained from the extended radicles of germinating seeds and fixed in the same manner. After fixing, the root tips were washed 24 hr. in running water and then dehydrated in the usual alcoholic solutions. Sections were cut 6 to 10 μ in thickness and stained in Haidenhain's iron-haematoxylin.

In a few cases, viz. *Medicago lupulina*, *M. sativa*, *M. media*, *Melilotus indica* and *Trigonella coerulea* pollen mother cells were studied for haploid chromosome numbers. For this purpose inflorescence buds 2 to 4 mm. long were killed by immersion for 1 to 2 min. in Carnoy's solution and then fixed by keeping for 24 hr. in Karpechenko's solution. This material was sectioned and stained as described above for root tips. The iodine gentian violet stain (Huskins, 22) was tried but did not prove as satisfactory as Haidenhain's iron—haematoxylin.

After root tips were taken the plants were transplanted into larger pots and grown to maturity in the greenhouse. Duplicates in some cases, especially of perennial forms, were transplanted into the field and grown to maturity under outdoor conditions. The flowering and matured plants were used for confirmation of species names.

The plants were identified by comparison with specimens in the Herbarium of the University of California, by the use of Taubert's (49) and Hegi's (15) keys, and McKee's and Ricker's (28) bulletin on "Non-perennial *Medicagos*". The specimens were checked for identification by Mr. Roland McKee, Senior Agronomist, Bureau of Plant Industry, Washington, D. C. Taubert's system of grouping the species into sections and sub-sections was adopted for purposes of this investigation.

A Leitz binocular microscope was used, which was equipped with 10×, 15× and 20× oculars and a 2 mm. objective with N.A. 1.30. Drawings were made at a magnification of 4,000 with the aid of a camera lucida.

Many attempts were made to intercross several of the species with a view to obtaining some further evidence of species kinship based on interspecific fertility and sterility. Numerous cross pollinations were made in each of the following crosses. In each case the first mentioned species was used as the female parent.

Medicago sativa	×	*Medicago lupulina*
Medicago sativa	×	*Medicago hispida*
Medicago sativa	×	*Medicago rugosa*
Medicago sativa	×	*Medicago falcata*
Medicago falcata	×	*Medicago lupulina*
Medicago falcata	×	*Medicago hispida*
Medicago rotata	×	*Medicago orbicularis*
Medicago rigidula	×	*Medicago sativa*
Medicago rigidula	×	*Medicago lupulina*
Medicago obscura	×	*Medicago sativa*
Medicago echinus	×	*Medicago sativa*
Medicago ciliaris	×	*Medicago orbicularis*
Medicago sativa	×	*Trigonella cretica*
Medicago ciliaris	×	*Trigonella coerulea*
Trigonella coerulea	×	*Medicago lupulina*

In cross pollinating, flowers on female parents were chosen, which were ready to "trip", but had not yet done so. All flowers but three or four of these were removed from the raceme. Those remaining were artificially tripped; the anthers were carefully extracted by means of fine-pointed forceps and the stray pollen was then removed. This was done, in some cases by washing out the pollen grains with a fine jet of water as recommended by Oliver (35). For this purpose a small hand sprayer with a rubber bulb and a fine nozzle was used. In other cases a modification of Oliver's method was used. This consisted in the removal of the pollen by means of the sharp corner of a piece of soft blotting paper used as a small brush. This was suggested to the writer by Southworth (45) who used an improvised brush made of gardener's raffia to clear away the pollen in emasculating flowers. A fresh piece of blotting paper was used for each flower. The pollen was carefully collected from the flowers of the male parent with a pair of sterilized fine-pointed forceps and deposited on the stigmas of the emasculated flowers. All

of the three or four emasculated flowers on one raceme were pollinated by pollen from the same male parent and an appropriate label was attached to the peduncle. Where crossing was done in the field the artificially pollinated flowers were covered with waxed-paper bags to prevent contamination by the visitations of bees and other insects; but where crossing was done in the greenhouse it was not thought necessary to protect the pollinated flowers in this way.

These efforts to hybridize were largely unsuccessful. However, in the following few cases pods were secured:

Medicago sativa	×	*Medicago lupulina*	4 pods
Medicago sativa	×	*Medicago hispida*	3 pods
Medicago sativa	×	*Medicago rugosa*	1 pod
Medicago rotata	×	*Medicago orbicularis*	1 pod
Medicago obscura	×	*Medicago sativa*	1 pod
Medicago ciliaris	×	*Trigonella coerulea*	2 pods

These apparently successful crosses may not all be genuine since it is possible that through faulty technique, self-fertilization may have occurred. The further study of these hybrid seeds has been deferred until a later date. The very large proportion of failures indicates that interspecific hybridization in *Medicago* is not readily accomplished.

Cytological Observations

The cytological observations will be presented first for *Medicago*, the species for the different sections according to Taubert's taxonomic classification being grouped together, second for *Melilotus*, and last for *Trigonella*.

Medicago

Section Lupularia

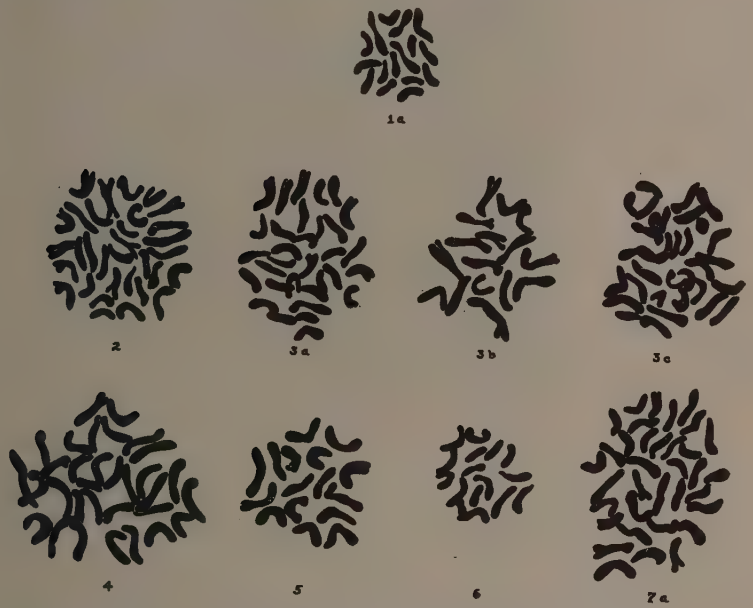
Medicago lupulina (L). This species only as representing this section was available for study. The somatic chromosome number is 16, Fig. 1a, and the number in the second metaphase of meiosis is 8. The somatic chromosomes range from about 1 to 2.7μ in length. There is some indication that a size classification among these might be possible. There appears to be at least one pair between 2.5 and 2.7 μ, another pair between 2 and 2.5 μ while the remaining 12 pairs range from 1.5 to 1.8 μ long. The general shape of the somatic chromosome varies from practically straight to falcate or strongly bent at the middle. The chromosome evidently has a median constriction which is presumably associated with the spindle-fibre attachment.

At the somatic anaphase the chromosomes assume in general a dumb-bell shape.

A seeming anomaly is the appearance of a nucleolar remnant in the somatic metaphase, Fig. 1e, (p. 29). In the case figured the 16 chromosomes are visible, two of which appear to have some connection or rather intimate relation with the nucleolar remnant.

Section Falcago

1. *Medicago sativa* (L). The somatic chromosome number is 32. Fig. 2. In length the chromosomes appear to vary but little, ranging from 2 to 3μ and no size classification is possible. They vary from slightly curved to strongly bent. No satellites were observed. The large number of rather small chromosomes made it impractical in this material to determine the more minute morphological details of the chromosomes.



Somatic chromosomes of Medicago, sections Lupularia and Falcago

FIG. 1A—*Medicago lupulina (L);* FIG. 2—*Medicago sativa (L);* FIG. 3A—*Medicago falcata (L);* FIG. 3B—*Medicago falcata (L);* FIG. 3C—*Medicago falcata (L) tetraploid chimeral area in a diploid plant;* FIG. 4—*Medicago media Pers. ("Grimm");* FIG. 5—*Medicago platycarpa (L) Trautv;* FIG. 6—*Medicago ruthenica Trautv;* FIG. 7A—*Medicago media-like plant.*

2. *Medicago falcata* (L). This species was represented in the material studied by three strains which have been designated I, II and III. In strains I and II the somatic chromosome number is 32, Fig. 3a, while in strain III it is 16, Fig. 3b. Chromosome length and shape features are practically the same in the first two strains, the length ranging from 1.7 to about 2.7μ, while in strain III the length is slightly greater varying from about 2 to 3.2μ. This difference, however, may not be significant. All strains have curved or falcate chromosomes. No size classification within any of the strains appears to be possible.

In strain III a tetraploid cell at metaphase was found in root tip material. Thirty-two chromosomes were counted, Fig. 3c. The cell was unusually large and the chromosomes appeared quite similar to those in the normal 16-chromosome cells of the strain but they were a little shorter, 1.7 to 2.8 μ.

No satellites were found, though there were cases where one of the chromosome arms appeared to be drawn out with a long constriction separating the chromosome end from the remainder of the chromosome. These ends were sometimes abnormally shaped and often were turned upward or downward from the common focal plane of the chromosome plate. These are thought either to be due to poor fixation or to small nucleolar remnants.

3. *Medicago media* Pers.("Grimm"). The somatic chromosome number is 32, Fig. 4, (p. 27). The chromosome length ranges from 2.2 to about 3.2μ and the shape varies from slightly curved to U- or V-shaped. No satellites were seen and no other minute morphological characters were visible.

4. *Medicago platycarpa* (L) Trautv. The somatic chromosome number is very probably 16, Fig. 5. One anaphase plate indicated 17 but several metaphase plates gave counts of 16. The length ranges from about 2.5 to 3.2μ with no classification based on size possible, and the shape is slightly curved to U-shaped. In anaphase the chromosomes are definitely shaped like dumbbells, thus suggesting median constrictions and equally two-armed chromosomes. In two plates there was an appearance of one or two large satellites, but these were uncertain and several good plates showed no evidence of satellites. Hence it is concluded that the appearance of these apparent large satellites is due to fixation irregularities and is not to be interpreted as indicating a normal feature of the chromosome complement.

5. *Medicago ruthenica* Trautv. The somatic chromosome number is 16, Fig. 6. The length ranges from 1.5 to 2.5 μ with no intranuclear size classification possible. The shape varies from slightly curved to decidedly bent presumably at the median constriction. No satellites were seen and no other minute morphological features were observable.

6. *Medicago media* Pers*. From this seed stock only one plant was secured. The somatic chromosome number appeared to be 35, Fig. 7a. Some metaphase plates showed 35 while a few seemed to show 34 or 36. One anaphase plate showed 35 chromosomes. This is, however, uncertain as the two anaphase plates were rather close together and counting was therefore difficult. Several meiotic plates were studied. First division metaphases showed 16 or 17 or 18 but the number appears most often to be 17, Fig. 7b. Second division metaphase plates showed 17 or 18.

The size of the somatic chromosomes varies from about 1.8 to 2.8μ in length and no classification based on size is practicable. In shape they are slightly curved to quite sharply bent and appear to have two equal arms separated by a median constriction. No satellites were observed.

In some second meiotic metaphase plates there appeared to be a certain amount of straying of chromosomes and in some second anaphase plates there was lagging and some belated chromosome separation, Fig. 7e. The tetrads

* *This seed stock was supposed to be Medicago prostrata, but the mature plant proved to have the morphological characteristics of Medicago media Pers.*

showed a few irregularities. In other cases one of the microspores was diminutive with an abnormally small nucleus. In one instance a microspore was abnormally large and binucleate. The pollen grains were also quite variable in size as shown in Fig. 7g. These cytological observations indicate that this plant was a hybrid. The high degree of sterility of the plant also indicates hybridity. On account of the evident hybrid nature of this plant these findings can have very little if any value in connection with the cytotaxonomic aspects of this investigation.

Summarizing for the above mentioned species in the *Falcago* section, *M. sativa*, *M. falcata* strains I and II and *M. media* have 32 chromosomes, *M. platycarpa*, *M. ruthenica* and strain III of *M. falcata* have 16 chromosomes while a *M. media*-like plant (probably a hybrid) had 35 chromosomes. The size and morphological features of the chromosomes in the various species are quite similar. *M. sativa*, Fig. 2, (p. 27) *M. media* ("Grimm"), Fig. 4, *M. falcata* strain III, Fig. 3b, and *M. platycarpa*, Fig. 5, have chromosomes which are a little larger than those of *M. falcata* strains I and II, Fig. 3a, and *M. ruthenica*, Fig. 6. The tetraploid cell in *M. falcata* strain III, Fig. 3c, had chromosomes similar to the latter group.

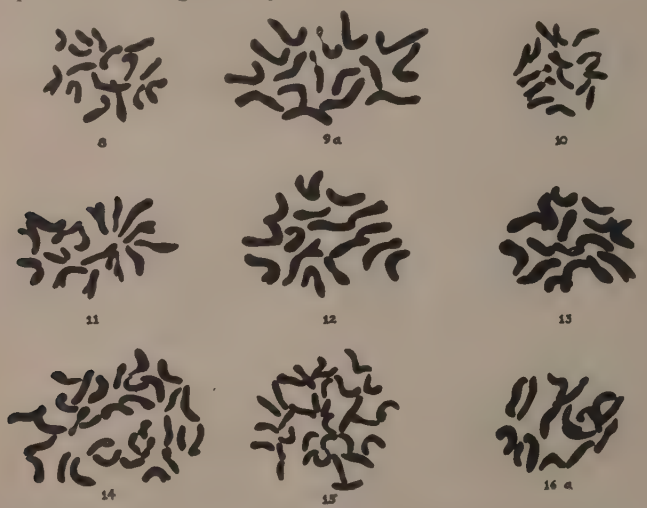
FIG. 1E—*Nucleolar remnant in a somatic cell of Medicago lupulina;* FIG. 7B—*Metaphase of first meiotic division in a Medicago Media-like plant;* FIG. 7E—*Lagging and belated chromosome separation in the second meiotic division in a Medicago media-like plant;* FIG. 7G—*Variable size of pollen grains in a Medicago media-like plant;* FIG. 17C—*Uncertain chromatin bodies attached to two somatic chromosomes in Medicago obscura. These may be large satellites or nucleolar remnants;* FIG. 27B—*A tetraploid chromosome set in a chimeral area in the root tip of a plant of Medicago hispida nigra;* FIG. 28B—*Attenuated median constriction in somatic chromosomes of Medicago laciniata;* FIG. 32B—*Dumb-bell shaped somatic anaphase chromosomes of Melilotus alba;* FIG. 32C—*Granular formation of somatic prophase chromosomes in Melilotus alba;* FIG. 34B—*Early anaphase of the first meiotic division in Trigonella coerulea.*

Section Spirocarpos—subsection Orbiculares

1. *Medicago orbicularis* All. The somatic chromosome number is 16, Fig. 8, (p. 30). In length the chromosomes vary from 1.5 to 2.5μ, but they are not classifiable on the basis of length, though in some plates one pair appears to be decidedly longer than the others. The shape is curved to sharply bent and

J-shaped. The chromosomes all appear fundamentally to be made up of two arms separated by a median constriction. This is borne out by the dumb-bell shape of the anaphase chromosomes. No satellites were observed in this species.

2. *Medicago carstiensis* Wulf. This species has 16 somatic chromosomes, Fig. 9a. The chromosomes vary in length from 2.5 to 4μ. They are large in comparison with most species of *Medicago*. In shape they vary from slightly curved through J-shaped to U- and V-shaped. One pair has large satellites, Fig. 9a. In the anaphase stage the chromosomes are dumb-bell shaped, similar to those represented in Fig. 32b, (p. 29).



Somatic chromosomes of Medicago, section Spirocarpos, sub-sections Orbiculares, Intertextae, Scutellatae, and Rotatae.

FIG. 8—*Medicago orbicularis All;* FIG. 9A—*Medicago carstiensis Wulf;* FIG. 10—*Medicago soleirolii Duby;* FIG. 11—*Medicago interlexta Mill;* FIG. 12—*Medicago echinus D.C.;* FIG. 13—*Medicago ciliaris (L) All;* FIG. 14—*Medicago scutellata (L) Willd.;* FIG. 15—*Medicago rugosa Desr.;* FIG. 16A—*Medicago rotata Boiss.*

3. *Medicago soleirolii* Duby. The somatic chromosome number is 16, Fig. 10. The length is from 1.2 to 2μ with no length classification possible. The chromosomes are falcate in shape at metaphase and show a tendency to assume the form of a dumb-bell in anaphase. Two chromosomes have large satellites which in some cases appeared definitely connected with their chromosomes, but in other cases the connecting threads were not clearly visible. In some metaphase plates nucleolar remnants were in evidence.

Summarizing for the three species in the subsection, *Orbiculares*, it may be said that all have 16 somatic chromosomes. *M. orbicularis* and *M. soleirolii* have small-sized chromosomes, 1.2 to 2.5μ, while *M. carstiensis* has much larger chromosomes ranging from 2.5 to 4μ in length. The general shape of the chromosomes in all three species is curved but *M. carstiensis* and *M. soleirolii* distinguish themselves from *M. orbicularis* by the possession of two large satellites in the chromosome complement of each.

Section Spirocarpos—subsection Intertextae

1. *Medicago intertexta* Mill. The somatic chromosome number is 16, Fig. 11. The length is from about 2 to 3μ. They vary from slightly curved to strongly bent in the metaphase and they are dumb-bell shaped in anaphase thus indicating median spindle fibre attachments. No satellites occur although in some plates there was some indication of secondary (4) or akinetic (12) constrictions.

2. *Medicago echinus* D.C. The somatic chromosome number is 16, Fig. 12. There appears to be some length difference among the chromosomes. One or two pairs are about 3μ long while the others range down to about 2μ. The shape is curved to strongly bent while the anaphase chromosomes show the usual dumb-bell shape. No satellites were observed. In the cortex a binucleate cell was found.

3. *Medicago ciliaris* (L) All. The somatic chromosome number is 16, Fig. 13. The length ranges from 2 to about 3μ and the shape is in general falcate. In the anaphase the chromosome is dumb-bell like but not so markedly as in many other species. No satellites were seen.

Summarizing for the three species representing subsection *Intertextae*, the somatic number is 16. The chromosomes range from 2 to 3μ in length. They are all falcate to strongly bent in shape, and all appeared to be without satellites.

Section Spirocarpos—subsection Scutellatae

1. *Medicago scutellata* (L) Willd. The somatic chromosome number is 32, Fig. 14. The counts varied from 30 to 33, but several plates appeared to have 32, and this is probably the correct number. The length ranges from 1.5 to 2.2μ with no intranuclear size classification possible. The chromosomes are falcate to U-shaped. No satellites were observed.

2. *Medicago rugosa* Desr. The somatic chromosome number is 32, Fig. 15. The length is from 1.2 to 1.7μ. Some plates appeared to have 31 and some 33, but 32 is probably the correct number. Chromosomes are slightly curved to U-shaped. No satellites were visible.

The chromosome features of these two species are quite similar. In both cases the somatic number is 32. Both have curved to strongly bent chromosomes of small size, viz. 1.2 to 2.2μ. The chromosomes of *M. rugosa*, however, appear to be somewhat the smaller.

Section Spirocarpos—subsection Rotatae

Medicago rotata Boiss. This subsection is represented by one species only. The somatic chromosome number is 16, Fig. 16a. The length varies from 1.5 to 2.5μ and the chromosomes do not appear to be classifiable on the basis of size. The chromosomes are falcate to strongly bent in shape. In many plates one or two chromosomes appeared to be pulled apart at the middle so that the two arms were separated or only very delicately adjoined. In some cases one arm appeared normal, i.e. elongated while the other was contracted longitudinally and thickened latterly, giving a round or oval shape. These contracted

arms were often above or below the general focal plane of the plate. It seems possible that these abnormal arms may have been due in some way to association with nucleolar remnants. In some cases they resemble large satellites. In anaphase the chromosomes assume the dumb-bell shape.

Somatic chromosomes of Medicago, section Spirocarpos, sub-sections Pachyspirae, Euspirocarpae and Leptospirae

FIG. 17A—*Medicago obscura Retz.*; FIG. 18—*Medicago littoralis Rhode*; FIG. 19A—*Medicago tuberculata aculeata*; FIG. 20—*Medicago rigidula (L) Desr.*; FIG. 21A—*Medicago murex (L) All*; FIG. 22A—*Medicago muricata (L) All*; FIG. 23—*Medicago arabica (L) All*; FIG. 24—*Medicago hispida denticulata Willd. Urban*; FIG. 25—*Medicago hispida confinis (Koch) Burnat*; FIG. 26—*Medicago hispida terebellum Willd. Urban*; FIG. 27A—*Medicago hispida nigra Willd. Burnat*; FIG. 28A—*Medicago laciniata Mill*; FIG. 29—*Medicago coronata Desr.*

Section Spirocarpos—subsection Pachyspirae

1. *Medicago obscura* Retz. The somatic number is uncertain but it appears to be either 16, 17 or 18. Several plates were counted giving each of these numbers; Fig. 17a represents one plate showing 18 chromosomes. In some plates two chromatic elements appeared to be connected end to end. Two

such double chromosomes were seen in each plate, Fig. 17c, (p. 29). If each of these is two chromosomes the number is 18 but if each is one chromosome the number is 16. An anaphase plate showed 18 chromosomes and it seems probable that the correct number is 18. However, counts in pollen mother cells will probably have to be made in order to determine accurately the chromosome number for this species. The somatic chromosomes are 1.2 to 2μ long and may be regarded as small in size. They are falcate in shape and appear to have no satellites.

2. *Medicago littoralis* Rhode. The somatic chromosome number is 16, Fig. 18. The length of the chromosomes ranges from 1.2 to 2.5μ. In shape they are falcate. Two large satellites were observed. These appeared to be nearly as large as one normal arm of the chromosome.

3. *Medicago tuberculata aculeata*. The somatic chromosome number is probably 16, Fig. 19a, though this is uncertain. In some plates there appear to be 16 chromosomes plus two large satellites or fragments. These latter elements are seemingly indeterminable. In many cases they appear to be detached from other elements and might be interpreted as small chromosomes or fragments. In other plates they appear to be connected by short threads to chromosomes. In shape the chromosomes are falcate as seems to be general for other species of *Medicago*. The length varies widely from 1.2 to 2μ. No size classification seems possible, however. Anaphase chromosomes are dumb-bell in shape.

One root tip was found in which two large tetraploid sectors with large cells on opposite sides of the root were observed. The alternating narrower sectors were diploid with smaller cells. This arrangement gave the cross section of the root a broad elliptical contour. The number of chromosomes in the tetraploid cells was not satisfactorily determinable. Some plates appeared to have 32 chromosomes while others appeared to have more. One plate gave a count of 34 elements. Some of these elements were small and may have been individual chromosomes or they may have been satellites.

4. *Medicago rigidula* (L) Desr. The somatic chromosome number is 14, Fig. 20. The size varies from 1.7 to 2.5μ in length. The chromosomes are two-armed with rather conspicuous median constrictions. In some cases the chromosome appears to be made up of two segments rather loosely joined end to end. This gives a dumb-bell appearance to some metaphase chromosomes. No satellites were observed.

5. *Medicago murex* (L) All. The somatic chromosome number is 16, Fig. 21a. The length is from 1.5 to 2.2μ, i.e. comparatively small. There may be one short pair from 1 to 1.5μ in length. The chromosomes in the early metaphase are falcate. In the late metaphase or very early anaphase they are distinctly two-armed with a very conspicuous median constriction which is sometimes greatly extended in length. Two large satellites are present. There appears to be intergradation from the large satellite condition to the attenuated medial constriction with the two arms more or less set apart.

6. *Medicago muricata* (L) All. The somatic chromosome number is 16, Fig. 22a, (p. 32). Chromosome length varies between 1.7 to 2.7μ. No size classification was possible. The chromosome shape varies from slightly curved to decidedly bent. There appears to be two large satellites, Fig. 22a. In some plates the large appendage appeared more like a slightly reduced arm of the chromosome. It would seem impossible to determine whether these are large satellites or primary arms connected by more or less elongated median constrictions with the remaining portions of the chromosomes. Anaphase chromosomes are decidedly dumb-bell shaped with no evidence of satellites.

Summarizing for the six species representing subsection *Pachyspirae*, the somatic chromosome number is variable. *M. obscura* has 16, 17 or 18. Both *M. littoralis and M. tuberculata aculeata* have probably 16. In the latter species tetraploid chimeral areas were found in one root tip. *M. rigidula* has 14. *M. murex* and *M. muricata* have 16. The chromosomes are all small ranging from about 1.2 to 2.7μ., while the shape of the chromosomes is in general falcate. They all appear to be fundamentally made up of two equal arms. In certain chromosomes, however, one arm is more or less reduced in size or modified in shape. In *M. obscura* there is some suggestion of end to end connection in certain chromosome couples. In *M. littoralis* one pair shows large satellites which approach in size one normal arm of the chromosome. In *M. tuberculata aculeata* there appears to be one pair of satellites or small chromosomes or fragments, the determination of which is difficult. They are probably satellites. In the tetraploid cells these small elements also occur. In *M. rigidula* several of the chromosomes display a very pronounced median constriction. No satellites were observed. *M. murex* has two chromosomes with large satellites. In some cases the satellite approaches in size the arm of a chromosome. *M. muricata* has a chromosome garniture somewhat similar to that of *M. murex*. At least one pair of chromosomes has large satellites or modified chromosome arms.

Section Spirocarpos—subsection Euspirocarpae

1. *Medicago arabica* (L) All. The somatic chromosome number is 16, Fig. 23, (p. 32). The chromosome length ranges from about 1.5 to 2.5μ. The shape is slightly curved to decidedly bent. One pair has large satellites, Fig. 23. There is also some suggestion of shape pairs in some plates. Occasionally a chromosome appears to be stretched out to an abnormal length with a long constriction usually submedian in position. It may possibly be a chromosome with its satellite, modified by poor fixation. The anaphase chromosomes are dumb-bell shaped.

2 (a). *Medicago hispida denticulata* Willd. Urban. The somatic chromosome number is 14, Fig. 24. The length varies from 1.5 to 3.5μ. There appear to be four long chromosomes 2.5 to 3.5μ and ten shorter ones ranging from 1.5 to 2.5μ in length. In shape the chromosomes are falcate with frequent median constrictions visible; no satellites were observed. Nucleolar remnants appeared in many metaphase plates.

2 (b). *Medicago hispida confinis*. (Koch) Burnat. The somatic chromosome number is 14, Fig. 25, (p. 32). The length is 1.7 to 3μ. The chromosomes vary from nearly straight to strongly bent. Two chromosomes have large satellites, the connecting threads of which are in some cases very faint or invisible. Anaphase chromosomes are dumb-bell like.

2 (c). *Medicago hispida terebellum* Willd. Urban. The somatic chromosome number is 14, Fig. 26. The chromosomes are falcate and vary in length from 1.5 to about 3μ. No satellites were seen.

2 (d). *Medicago hispida nigra* (Willd) Burnat. The somatic chromosome number is 14, Fig. 27a. The chromosome length is 1.8 to 3.5μ, One or two pairs appear to be rather decidedly bent. Two chromosomes have large satellites attached, Fig. 27a. The thread connecting the satellite is sometimes only faintly to be seen. One tetraploid cell at metaphase was seen, Fig. 27b, (p. 29). Twenty-eight chromosomes were counted in this tetraploid cell, though the number is somewhat uncertain. These chromosomes appear to be a little smaller than in the diploid cells, viz. 1.7 to 2.8μ.

Summarizing the subsection *Euspirocarpae*, the somatic number is 16 for *M. arabica*, and 14 for the four varieties of *M. hispida*. Chromosome size is quite variable. Those for *M. arabica* are smallest—1.5 to 2.5μ in length. For *M. hispida confinis* and *M. hispida terebellum* the length ranges a little higher, viz. 1.5 to 3μ. *M. hispida denticulata* has about 10 small chromosomes about the same size as those of *M. arabica* while about four are longer—2.5 to 3.5μ. *M. hispida nigra* has the longest chromosomes of any species or variety representing this subsection. These chromosomes range from 1.8 to 3.5μ in length. In a tetraploid cell of *M. hispida nigra* chromosomes were somewhat smaller ranging from 1.7 to 2.8μ. In general, the chromosome shape in all these species and varieties is falcate. The most pronounced difference is the presence of a pair of large satellites in *M. arabica*, *M. hispida confinis* and *M. hispida nigra*, while in *M. hispida denticulata* and *M. hispida terebellum* no satellites were found.

Section Spirocarpos—subsection Leptospirae

1. *Medicago laciniata* Mill. The somatic chromosome number is 16, Fig. 28a, (p. 32). The size is variable from 1.7 to 3μ in length. The shape is falcate. In many plates one pair appears to have a tendency for the arms to draw apart leaving a long constriction or leaving no visible thread connection, Fig. 28b, (p. 29). One of these arms or segments sometimes has the appearance of a large satellite. Three apparent tetraploid cells at metaphase were found in the epidermal region of three successive sections but they were not in such condition as to make counting possible.

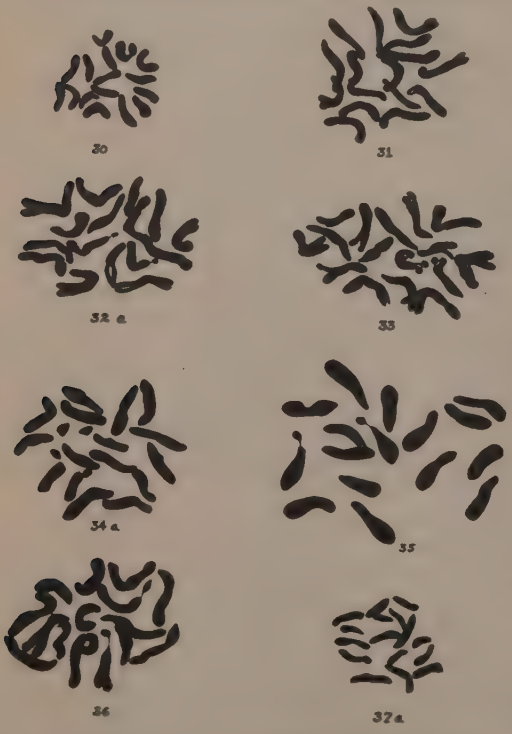
2. *Medicago coronata* Desr. The somatic number is probably 16, Fig. 29, (p. 32). The length ranges from 1.7 to 2.2μ. A few plates showed 17 chromosomes and in some cases the individual chromosomes could not be defined with certainty. The chromosomes are falcate in shape and no satellites could be observed with certainty.

Summarizing for subsection *Leptospirae* the somatic chromosome number is probably 16 for both species. In length the chromosomes range from 1.7 to 3μ for *M. laciniata* and from 1.7 to 2.2μ for *M. coronata*. The shape is, in general, falcate. In *M. laciniata* there were appearances suggesting large satellites but these were thought to represent modified chromosome arms attached by means of attenuated median constrictions. No satellites could be observed with certainty in *M. coronata* and individual chromosomes were difficult to define.

Melilotus

Section Campylorytis

Melilotus sulcatus Desf. The somatic chromosome number is 16, Fig. 30. The chromosome size is from 1.5 to 2.2μ in length. The shape is falcate with no possibility of chromosome classification. No satellites were observed.



Somatic chromosomes of Melilotus and Trigonella

Fig. 30—*Melilotus sulcatus Desf;* Fig. 31—*Melilotus officinalis (L) Medikus;* Fig. 32a—*Melilotus alba Medikus;* Fig. 33—*Melilotus indica All;* Fig. 34a—*Trigonella coerulea (L) Ser;* Fig. 35—*Trigonella foenum graecum (L);* Fig. 36—*Trigonella foenum graecum (L);* Fig. 37a—*Trigonella cretica (L) Desr.*

Section Plagiorytis

Melilotus officinalis (L) Medikus. The somatic chromosome number is 16, Fig. 31. The chromosome size is from about 3 to 4μ in length. The shape is long, slender and curved to decidedly bent, with no possibility of chromosome classification. No satellites appeared. The chromosomes have median spindle fibre attachments.

Section Coelorytis

1. *Melilotus alba* Medikus. The somatic chromosome number is 16, Fig. 32a. The chromosome size is about 3 to 4μ in length. The shape is curved to U- and V-shaped. Two large satellites occur. The anaphase chromosome is dumb-bell in shape, Fig. 32b, (p. 29) and hence the spindle fibres probably have median attachment. The prophase chromosome appears to be made up of a chain of granules, Fig. 32c, (p. 29) and the metaphase chromosome seems to be formed by a shortening of the prophase chromosome chain through a coalescence of the granules.

2. *Melilotus indica* All. The somatic chromosome number is 16, Fig. 33. The length varies from 2.5 to 3.8 μ. The shape is curved to V-shaped. One pair appears to have two constrictions in each chromosome, Fig. 33. Meiotic first metaphase plates show eight chromosomes, which confirms the somatic determination.

Summarizing the four species representing the genus *Melilotus*, all have 16 somatic chromosomes and *M. indica* has eight as the haploid number. *M. sulcatus* has chromosomes 1.5 to 2μ in length while the other three species have decidedly longer chromosomes, though M. *indica* may have slightly shorter chromosomes than *M. officinalis* and *M. alba*. All four species have curved to decidedly bent chromosomes. *M. alba* is, however, distinguished by the possession of two large satellites.

TRIGONELLA

Section Eutrigonella—subsection Capitatae

Trigonella coerulea (L) Ser. The somatic chromosome number is 16, Fig. 34a. In the early anaphase of the first meiotic division eight dumb-bell shaped chromosomes were seen, Fig. 34b, (p. 29). The chromosome size is from about 2.2 to 3.2 μ in length. The shape is slightly curved to falcate and there are four large satellites or fragments. These in many cases look like detached fragments or heads of rod-like chromosomes. At any rate they appear to be a constant feature.

Section Eutrigonella—subsection Gladiatae

1. *Trigonella foenum graecum* (L). The somatic chromosome number is 16, Fig. 35. The size of the chromosomes is from 2.5 to 3.3μ in length. The shape is from rod-like to falcate. Two chromosomes have large satellites. All the chromosomes of this species appear to be thicker and more blocky than those studied in *Medicago*. In this case it appears scarcely to be a case of less destaining.

2. *Trigonella foenum graecum* (L). The somatic chromosome number is 16, Fig. 36. The length varies. There appear to be about eight which range from 2.5 to 3.5μ while the other eight range from 4.5 to 5μ. The chromosomes are long and relatively slender. They are curved and coiled, the ends extending somewhat above or below the general focal plan of the chromosome plate. Two large satellites occur.

This material was at first thought to be another species, but later proved to be *T. foenum graecum* and morphologically indistinguishable from the preceding stock. The chromosome complexes are seen to be quite similar. In both cases the chromosomes are large and two satellites are present. In this material, however, some of the chromosomes appear to be somewhat longer than those of the *T. foenum graecum* stock first mentioned.

Section Pocockia—subsection Samaroideae

Trigonella cretica (L) Desr. The somatic chromosome number is probably 16, Fig. 37a. In some plates the arms of one or two chromosomes appeared to

be separated by an elongated constriction or to be entirely disconnected. The number in these cases seems to be 17 or 18. But the smaller elements are probably disconnected chromosome arms. The anaphase plates showed 16 dumb-bell chromosomes and therefore 16 is likely the correct somatic number. The chromosome size ranges for a few small ones from 1.2 to 1.5μ and for the larger ones from 1.7 to 2.5μ in length. The chromosome shape is curved or falcate. No satellites were observed.

Summarizing the results found for the three species of *Trigonella*, the somatic chromosome number is 16, the number in *T. cretica* being a little uncertain. In size the chromosomes of *T. coerulea* and *T. foenum graecum* are about the same, i.e., from 2.5 to 3.7μ long, *T. coerulea* being slightly shorter, while those of *T. cretica* are decidedly smaller, viz., 1.2 to 2.5μ. *T. coerulea* has four large satellites or detached fragments. *T. foenum graecum* has two fairly large satellites while *T. cretica* probably has no satellites. *T. cretica* appears to possess one or two pairs of chromosomes, the halves of which tend to disconnect at the median constriction with the result that the halves resemble small chromosomes.

Summary statement of cytological observations

In the following statement the chromosome numbers found by other workers are included with those found by the writer. The former are put in separate columns. At the end of the *Medicago* list are mentioned a few species of *Medicago*, all of which have been placed in section *Spirocarpos* by Ghimpu, but their subsection relations were not given and hence they have been grouped together. The symbols, L, l, M, m, S, s, refer to lengths of the chromosomes, designating relative length ranges respectively from about 5μ down to 1μ. The bracketed symbols refer to the length classes of chromosomes in the species and their numbers. The abbreviations "sat" and "T" refer respectively to the occurrence of satellites and of tetraploid cells or sectors in otherwise diploid root tips.

TABLE I

CYTOLOGICAL OBSERVATIONS

Genus Medicago	Fryer		Figure number	G[1]	K[2]
	n	2n		2n	n
Section—*Lupularia*					
M. lupulina (L)	8	16 (2m+2S+12s)	1a	16	8
Section—*Falcago*					
M. sativa (L)		32(32m)	2	32	16
M. falcata (Strains I, II)		32(32S)	3a		16
M. falcata (Strain III)		16(16m) T(32)	3b		
M. media "Grimm" Pers.		32(32m)	4		
M. platycarpa (L) Trautv.		16(16M)	5		8
M. ruthenica Trautv.		16 (16S)	6		
M. media		35 (35m)	7a		
Section—*Spirocarpos*					
Subsection *Orbiculares*					
M. orbicularis All.		16(16S)	8	16	
M. carstiensis Wulf.		16(16,1+M)2 sat.	9a		
M. soleirolii Duby.		16(16s) 2 sat.	10		
Subsection—*Intertextae.*					
M. intertexta Mill.		16(16m)	11		
M. echinus D.C.		16(2M+14m)	12	16	
M. ciliaris (L) All.		16(16m)	13		
Subsection—*Scutellatae.*					
M. scutellata (L) Willd.		32(32S & s)	14	32	
M. rugosa Desr.		32(32S & s)	15		
Subsection—*Rotatae.*					
M. rotata (Boiss)		16(16S)	16a		

[1] *Ghimpu (14).* [2] *Karpechenko (1925) see Tischler (54).*

	Fryer	Figure number	G	K
	2n		2n	n
Subsection—*Pachyspirae.*				
M. obscura Retz.	16 or 18 (16 or 18s)	17a		
M. littoralis Rhode.	16(16S) 2 sat.	18		
M. tuberculata aculeata	16(16s) 2 sat. T(32)	19		
M. rigidula (L) Desr.	14(14S)	20	16	
M. murex (L) All.	16(16S) 2 sat.	21		
M. muricata (L) All.	16 (16S) 2 sat.	22		
Subsection—Euspirocarpae.				
M. arabica (L) All.	16(16S) 2 sat.	23		
M. hispida denticulata Willd. Urban.	14(14m & S)	24		
M. hispida confinis (Koch) Burnat.	14(14m & S) 2 sat.	25		
M. hispida terebellum (Willd) Urban.	14(14m & S)	26		
M. hispida nigra (Willd) Burnat.	14(14m & S) 2 sat. T.	27		
Subsection—*Leptospirae.*				
M. laciniata Mill.	16(16m & S) T.	28		
M. coronata Desr.	16(16S)	29		
Section *Spirocarpos.*				
M. disciformis			16	
M. minima			16	
M. maculata			16	
M. spaerocarpa			16	
M. tornata			16	
M. fenoreana			16	
M. truncatula			16	
M. helix			16	

TABLE I (*Continued*)

	Fryer 2n	Figure number	Elders 2n	Castetter n
Genus Melilotus.				
Section—*Campylorytis.*				
M. sulcatus Desf.	16(16S)	30		
Section—*Plagiorytis.*				
M. officinalis (L) Medikus	16(16,l) slender	31	16	
Section—*Coelorytis.*				
M. alba Medikus.	16(16,l) 2 sat.	32a	16	8
M. indica All.	16(16,l & M)	33		
Genus Trigonella.				
Section—*Eutrigonella.*				
Subsection—*Capitatae.*				
T. coerulea (L) Ser.	16(16m) 4 sat.	34a		
Subsection—Gladiatae.				
T. foenum graecum (L)	16(16m) thick, 2 sat.	35		
T. foenum graecum (L)	16(16L&M)coiled, 2sat.	36		
Section—*Pocockia.*				
Subsection—*Samaroideae.*				
T. cretica (L) Desr.	16(16S & s)	37a		

Discussion

A casual survey of the taxonomic classification by Taubert and the cytological findings of this investigation indicates a general agreement between the two, although there are some discrepancies which will be discussed later.

General morphology of the chromosomes

The somatic metaphase chromosomes in *Medicago*, *Melilotus* and *Trigonella* are variable in shape from straight or falcate to strongly bent, U- or V-shaped. These variations, however, are not thought to be significant. Fundamentally they all appear to be two-armed, each with a median constriction which in all probability marks the position of the spindle fibre attachment. In many cases the chromosome appears to be unequal-armed, one arm appearing to be shorter or smaller than the other. This in many instances is likely due to the chance position or orientation of the chromosome with reference to the general plane of the chromosome plate. This general structure of the chromosomes agrees with Heitz's view (1928) as referred to by Hollingshead and Babcock (21) that the two-armed nature of the chromosome is a universal characteristic. Heitz is also credited by these authors with the view that the equal-armed chromosome is the primitive one. If this view is correct, the chromosomes in *Medicago*, *Melilotus* and *Trigonella* are to be regarded for the most part as primitive chromosome forms. In some species of these genera, however, the original primitive chromosome has become modified most conspicuously by the formation of large satellites. Also in a few species as, for instance, *Melilotus indica*,

Fig. 33, (p. 36) there is some evidence of a secondary constriction as well as the primary one, while in *Trigonella foenum graecum* one arm of several of the chromosomes appears to have become reduced in size. The fundamental two-armed nature of the chromosomes is further emphasized by the occurrence of dumb-bell chromosomes noted in the somatic anaphase in several of the species studied, e.g. *Melilotus alba*, Fig. 32 b, (29).

The chromosome size varies considerably among the different species of all three genera ranging from about 1-2μ in length to 3 or 4μ for species of *Medicago*, from about 1.5 or 2μ to 3 or 4 μ for species of *Melilotus* and from about 2 to about 5μ for *Trigonella*. Of the three genera the chromosome length variation appears to be greatest in *Trigonella*. In general the chromosomes of *Trigonella* are larger than those of *Medicago*, while those for *Melilotus* appear to average intermediate in size between *Trigonella* and *Medicago*.

Chromosome numbers

The somatic chromosome numbers found in *Medicago* are 14, 16 and 32, and possibly 18 for *M. obscura* and 35 for a *M. sativa*-like type. The last two numbers ought not to be included here, however, since 18 for *M. obscura* is uncertain and 35 for the *M. sativa*-like type was obtained from one plant which showed evidences of being either a hydrid plant or a chromosome variant derived from some other form. The numbers 14, 16 and 32 do not present a wide range of chromosome numbers for the genus *Medicago*. The various sections and subsections of the genus are fairly well represented in this study and the results would indicate that for the most part the numbers 14, 16 and 32 characterize the majority of the species. With this limited number series, it might seem that the species are all comparatively closely related. However, the chromosome number for the four species of *Melilotus* and the four species of *Trigonella* is 16 while most of the species of *Trifolium* so far studied have 2n = 14, 16 or 32, Tischler (54), and it might therefore seem on the basis of chromosome number that the four genera, *Medicago*, *Melilotus*, *Trigonella* and *Trifolium* are rather closely related. But Tischler (54) reports some species of *Vicia*, *Lens*, *Lathyrus* and *Pisum* as having 14 somatic chromosomes. These genera are obviously not very similar morphologically to *Medicago* and hence the same chromosome numbers, viz. 14, in these groups cannot be regarded as indicative of certain close genetic relationship. The occurrence of 16 chromosomes in certain species of *Medicago*, *Melilotus*, *Trigonella* and *Trifolium* is, however, suggestive of genetic relationship, and it may be that 16 is the number for an ancestral form from which these four genera have evolved.

In some taxonomic groups (sections or subsections) of *Medicago* the chromosome number is constant for the species studied, while in other groups the chromosome number varies. In subsections *Orbiculares*, *Intertextae*, *Scutellatae* and *Leptospirae* of section *Spirocarpos* the chromosome numbers are constant and in all but *Scutellatae* which has 32, the number is 16. In section *Falcago* only species with 16 and 32 chromosomes were found, leaving the *M. sativa*-like plant out of account as it should be for reasons mentioned above. The occurrence of these numbers within a section does not necessarily imply

lack of rather close relationship since tetraploidy is recognized as a common process which may give rise to new forms. Hollingshead and Babcock (21) think that 16-chromosome species of *Crepis* may have arisen from the 8-chromosome species by a process of doubling of the entire chromosome complement. Tetraploid plants have occasionally been found to occur in populations of diploids. Navashin (3) found that 0.5% of a total population in *Crepis tectorum* consisted of triploids and tetraploids. It seems probable that under natural conditions tetraploids thus arising may occasionally survive to initiate a new species. Considerations of this kind suggest that the 32-chromosome species of *Medicago* in the *Falcago* section may have arisen from the 16-chromosome species by tetraploidy, and that they are therefore rather closely related. The 16-chromosome species of this section are, however, more likely to be closely related among themselves as are also those of the 32-chromosome species among themselves, than are the two series to each other. On the basis of chromosome number it would seem that this section ought to be divided into two subsections, the 16-chromosome forms being grouped in one subsection and the 32-chromosome forms in the other.

Chromosome numbers and the annual or perennial habit in Medicago

The cytological results indicate no relation between species with higher chromosome numbers and the perennial habit in species of *Medicago*. *M. sativa*, *M. falcata* and *M. media* are perennial with 32 chromosomes, while *M. scutellata* and *M. rugosa* are annuals with 32 chromosomes. Conversely *M. platycarpa*, *M. ruthenica* and *M. carstiensis* are perennials with 16 chromosomes while *M. orbicularis*, *M. soleirolii*, *M. intertexta*, etc. are annuals with 16 chromosomes. This situation suggests that chromosome number has no relation to the life duration period. The life period is, therefore, probably determined by genetical point factors resident in certain chromosomes.

Races in Medicago falcata

Of the three lots of plants designated *Medicago falcata*, two, strains I and III, were prostrate in their habit of growth and bore falcate-shaped pods, while strain II was erect in growth habit but had falcate pods. Strain III had the morphological characteristics of true *M. falcata* though its chromosome number is 16 instead of 32, the number found for the other two lots of *M. falcata*. This indicates that cytologically at least, there are two different races within the species *M. falcata*, one with 32 chromosomes and one with 16 chromosomes. Previous mention has been made of the great morphological variability which Oakley and Garver (34) found in this species. It would seem in the light of the cytological findings here reported that the genus *Falcata* ought to be divided into two species at least, one species having 16 and the other having 32 chromosomes. However, further study of the cytological features of the different variations and types ought to be made with a view to a better classification of the forms now included within this species.

Hybrid origin of Medicago media

Cytological study has shown the chromosomes of *Medicago sativa*, *M. falcata* (32-chromosome form) and *M. media* to be the same in number and very similar morphologically, the only difference being that the chromosomes of *M. media*, Fig. 4, (p. 27) appear to be a little larger than those of *M. sativa*, Fig. 2, and *M. falcata*, Fig. 3a. Too much significance should not be attached, however, to this slight difference in size since chromosome sets vary somewhat in size in different tissues of the same root tip and in different root tips of the same plant. There is therefore no reason, from cytological considerations, to doubt the origin of *M. media* from interspecific hybridization between *M. sativa* and *M. falcata*.

Relations of Medicago lupulina

The cytological features of *Medicago lupulina* do not give any evidence as to the true genetical relationship of the species to other species of *Medicago* except that its chromosome number, 16, suggests its relation to the 16-chromosome species. The chromosomes of this species appear to be classifiable on the basis of comparative length. One pair is relatively long, six pairs are very short, while one pair is intermediate. Further study may disclose the significance of this size variation.

M. lupulina is annual in that it will set and mature seed in the first season of its growth. With mild winter conditions it will produce a second setting of mature seed the second season, and in this it displays a tendency towards the perennial habit. *M. media*, at least, has also been observed to set seed during the first and subsequent seasons of its life. *M. lupulina* therefore appears to resemble the perennial species in its perennial tendencies. Morphologically, however, it is quite distinct from other species of *Medicago*, particularly in respect to its pods and their single seeds.

In view of the lack of relation in *Medicago* between chromosome number and life duration, referred to above, it would seem probable that the annual and perennial tendencies in *M. lupulina* are controlled primarily by genic factors resident in some of the chromosomes and are not related to the number or morphological features of the chromosomes.

Relations of Medicago carstiensis

In the taxonomic section of this paper it is pointed out that *M. carstiensis* is associated with other perennial species, as *M. arborea*, *M. sativa*, *M. hybrida* and *M. prostrata* by Hegi (15) and with the annual species *M. orbicularis* and *M. soleirolii* by Taubert (49). Hegi apparently based his taxonomic placing of this species partly on morphological features and partly on the perennial habit of the species. Taubert based his placing entirely on morphological considerations. The cytological findings do not appear to give any definite evidence in regard to the relation of this species to others. It is similar cytologically to *M. soleirolii* in that both species have a pair of chromosomes with satellites. In other respects *M. carstiensis* appears to be distinct. The

chromosomes are longer than those found in any other species of *Medicago* and they are decidedly larger than the chromosomes of *M. orbicularis* or of *M. soleirolii*, Fig. 8, 9a and 10, (p. 30). The size differences here are great enough to be regarded as probably significant. Six of the chromosomes of *M. carstiensis*, Fig. 9a, appear to be unequal-armed while the others, apart from the two chromosomes with satellites, appear to be equal-armed. This unequal-armed condition may be significant. Cytologically therefore *M. carstiensis* appears to show no close relationship with any other species of *Medicago* yet studied. This view is supported by the stononiferous growth habit of the species, a character not observed in any other of the species studied.

Relations of Medicago obscura, M. rigidula and M. arabica

The chromosome number for *M. obscura* was not definitely determined. It is either 16 or 18. If it is 16 this species corresponds with most of the other species in subsection *Pachyspirae*. Otherwise it is a quite distinct species exhibiting cytologically no relationships with other species of *Medicago*. Further work will doubtless disclose the true chromosome number for this species.

Ghimpu (14) reported the chromosome number of *Medicago rigidula* as 16 but the material worked with in this investigation was found to have only 14 chromosomes. The difference in these determinations is possibly attributable to different varieties of the species being used in the two cases, although it is scarcely to be expected that two varieties of one species would have different chromosome numbers. It may be, however, that taxonomists have designated two forms as varieties of the same species, each of which ought in reality to be given the status of a distinct species. It may incidentally be pointed out that the *M. rigidula* material used in this investigation was identified as true *M. rigidula* by the late Professor P. B. Kennedy, University of California.

The cytological evidence indicates on the basis of chromosome number that *M. rigidula* does not belong to subsection *Pachyspirae* as placed by Taubert (49), in which four other species were found to have 16 chromosomes. Moreover these four other species each have a pair of chromosomes with satellites, while in *M. rigidula* no satellites were observed. Based on chromosome number it would seem that *M. rigidula* might more appropriately be grouped with four varieties of *M. hispida* in subsection *Euspirocarpae* all of which have 14 chromosomes. The external morphological characters of *M. rigidula* are not so different from those of the species in the *Euspirocarpae* subsection as to make unreasonable its association with the species of this subsection.

Medicago arabica has been placed by Taubert (49) in section *Spirocarpos* subsection *Euspirocarpae*. But the somatic chromosome number has been determined as 16 and it therefore differs from the varieties of *M. hispida* studied as representing this group, which have 14 somatic chromosomes. Its morphological characters would not seem to preclude it from being transferred to subsection *Pachyspirae* where it would be associated with 16 chromosome species. So far as the two satellites of *M. arabica* are concerned it may well

be related to the species of either subsections *Pachyspirae* or *Euspirocarpae*. The cytological findings would therefore indicate that *M. rigidula* and *M. arabica* ought to be exchanged in their taxonomic group relations as arranged by Taubert.

Varieties of Medicago hispida

The four varieties of this species which were studied, viz. *M. hispida denticulata*, *M. hispida confinis*, *M. hispida terebellum* and *M. hispida nigra*, are quite similar morphologically except for the pod characters in which they exhibit considerable difference. The chromosomes for these varieties are 14 in number and they are similar in size, but *M. hispida confinis* and *M. hispida nigra* differ from the other two varieties in the possession of two chromosomes with satellites. This situation is difficult to explain. Hollingshead and Babcock (21) point out an analogous situation in *Crepis*, viz. the occurrence of races within a species which differ in the shape of one chromosome. It may be that varieties differing in chromosome morphology as in this case are not really closely related although they may be similar in external morphology. Perhaps the forms with deviating chromosome morphology ought really to be regarded as distinct species. Or it may be that these chromosomal differences represent changes incidental to the transformation of a variety into a new species.

Tetraploid chimeral areas

In four species, viz. *Medicago falcata* (16 chromosome form), *Medicago tuberculata aculeata*, *Medicago hispida nigra* and *Medicago laciniata*, groups of vegetative cells containing tetraploid chromosome complements were found. Similar polyploid chimeral areas have been found by other investigators in other genera, particularly in *Crepis*. Babcock and Navashin (3) make reference to polyploid areas found by M. Navashin in *Crepis tectorum* and *C. dioscoridis*, and by Miss Hollingshead in *C. bureniana*. They also mention the common occurrence of diploid areas in haploid *C. capillaris* as found by Miss Hollingshead and M. Navashin.

The origin of these polyploid chimeral areas is not known. Two theories have been advanced. M. Navashin (31) suggested that they arise as a result of successive divisions without cytokinesis and the separation of daughter nuclei. S. Navashin (1928) as reported by Babcock and M. Navashin (3) suggested the idea of the fusion of nuclei or cells. These two suggested explanations do not seem necessarily to be opposed to each other. It may be that two daughter nuclei are retained within the mother cell walls by failure of cytokinesis. Binucleate cells have been observed in root tips. In *Medicago echinus* a binucleate cell was found in the cortex of the root tip. It is conceivable that the nuclei of such a cell may fuse in the following cell division, one instead of two spindles being formed. Hence, failure of cytokinesis followed by nuclear fusion may possibly be the process by which tetraploid vegetative cells are initiated.

Blakeslee and Belling (5) report that several cases in *Datura Stramonium* were found, chiefly after treatment with cold in which single branches on otherwise normal 2n plants were tetraploid. If the cold treatment initiated the tetraploid growth in *Datura*, it would seem possible that the tetraploid chimeras in *Medicago* might also have been caused by watering the growing plants in a warm greenhouse with comparatively cold water.

Nucleolar remnants

In several species chromatic bodies were observed at the somatic metaphase, Fig. 1e, (p. 29) and 17c, along with the matured chromosomes. In some instances these were two in number and they were apparently connected by a thin thread each to a chromosome. They were sometimes irregular in shape and turned upward or downward from the general plane of the chromosomes. In *Medicago obscura* it was difficult to make accurate counts of the chromosomes because of the difficulty of determining whether two elements were chromosomes or nucleolar remnants, Fig. 17c. In this figure it is difficult to determine whether the four elements in the upper left are four chromosomes or two chromosomes with two attached nucleolar remnants. Sometimes these so-called nucleolar remnants are small and appear to resemble two large satellites. These observations would seem to support the contention that the nucleolus during the prophase functions in the formation of the metaphase chromosomes. Zirkle (61) describes the progressive changes in the nucleolus during the prophase of mitosis in root tips of *Zea mays*. He states that after the spireme is formed the nucleolus becomes pear-shaped with the small lobe attached to the spireme. As the spireme flattens out in the equatorial plane the nucleolar material flows into it. The smaller lobe is drawn out and split into two, giving the nucleolus two connections with the spireme. Zirkle also points out that as the nucleolus loses its material it becomes rod-shaped and finally lies at right angles to the plane of division. It then is drawn out and constricted into two; the two fragments later round up and migrate to the poles. It seems possible that the so-called nucleolar remnants observed in *Medicago* correspond to these two fragments found by Zirkle in *Zea mays*. At any rate it seems evident that the nucleolar remnants have some functional and morphological relation to the maturing somatic chromosomes in some species of *Medicago* at least.

Anaphase chromosomes

The somatic anaphase chromosome was found to assume a rather peculiar shape in all three genera studied. The metaphase chromosome splits, and apparently very quickly the two arms of the daughter chromosome shorten until each is more or less globular in shape. The two arms lie side by side, separated by the median constriction, so that each anaphase chromosome has more or less the appearance of a dumb-bell with the connecting bar so shortened that the two bells are nearly in contact with each other. These are the dumb-bell anaphase chromosomes so frequently referred to in the Cytological Observations given above.

This type of anaphase chromosome appears to be unusual, as no mention of such a type could be found in standard text books on Cytology nor was any type of this kind discovered in any of the cytological literature reviewed. The significance of this form of anaphase chromosome is not evident; however, the reduced size facilitates counting the chromosomes when the two anaphase plates are sufficiently separated to prevent confusion of chromosomes in the two plates.

General Conclusions

When the investigation here reported was undertaken, hopes were entertained that the application of cytological methods to the genus *Medicago* might result in the acquisition of knowledge which could be utilized to help clear up some of the taxonomic difficultices in this genus, and which incidentally would be an aid in the more intelligent pursuit of plant breeding projects involving species included in the genera studied. These hopes have been partially realized in that new evidence has been obtained concerning some species and variety relationships which appear previously not to have been fully understood and also in that the information secured will be useful to plant breeders who contemplate interspecific hybridization between the species studied.

The results so far obtained indicate that the cyto-taxonomic method is applicable to these genera and that cytological investigations of other species and more intensive studies of certain species and varieties may be expected to yield results which will be valuable in taxonomic and other biological connections.

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SOIL AS A SOURCE OF INFECTION OF HONEY BY SUGAR-TOLERANT YEASTS[1]

By A. G. Lochhead[2] and Leone Farrell[3]

Abstract

Examination of soil from different locations, extending over a twelve-month period, for the presence of sugar-tolerant yeasts capable of fermenting honey, showed that only in the case of apiary ground was the soil regularly infected by such organisms. Ordinary field soil is not to be considered a primary source of infection of honey, sugar-tolerant yeasts not being regarded as members of the normal soil microflora. Sugar-tolerant yeasts, in apiary soils, are able to remain viable through the winter in frozen soil, but are cold-enduring rather than psychrophilic. From the soil 166 cultures of sugar-tolerant yeasts were isolated and classified. These were found to represent seven types considered as six species with one sub-species, three being types found in fermented honey. A description of the yeasts is given.

Introduction

In the course of previous experiments reported from this laboratory (6) dealing with the contamination of honey by sugar-tolerant yeasts capable of causing fermentation, investigations were made of various sources of infection. Studies of flowers commonly visited by bees during the honey flow resulted in the isolation of 11 different yeasts capable of fermenting high concentrations of honey, of which two proved to be identical with species found by the authors in question in fermented honey, while a third type was found to be similar to *Torula mellis* isolated by Fabian and Quinet (2) likewise from fermented honey.

Concurrent studies of nectar from hives throughout the season yielded results indicating an early and constant infection, though the yeast types isolated were fewer in number than from floral nectar, four in all being found. Of these three were identical with species found in flowers. Furthermore, from honey tanks, containers and air of the honey-extracting house five sugar-tolerant yeasts were isolated, all of which were recognized as types previously found in flowers, while three were, in addition, identified with types found in fermented honey samples.

In view of the comparatively wide distribution of sugar-tolerant yeasts noted in the work referred to, it was decided to extend the investigation to the soil, and consequently the studies here reported are concerned with an examination of soil from different locations, with the object of learning to what extent soil may be regarded as a natural habitat of honey-fermenting yeasts.*

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Historical

A number of studies are recorded in the literature dealing with the occurrence of yeasts in soil and describing types isolated by various authors, although, as far as we are aware, such investigations have not been concerned with the examination of soil for the presence of yeasts capable of fermenting honey, nor have tests been made, apparently, to determine whether the yeasts isolated were in any degree sugar-tolerant.

Many of the earlier studies deal with an examination of soil for yeasts occurring on fruits and for types which might be concerned with the brewing and wine-making industries. As such investigations are of but indirect concern to the question of the presence of honey-fermenting yeasts in soil, a detailed résumé of the literature in question seems out of place, and the reader may be referred to Guilliermond (3), Jörgensen (5), and to the paper by Starkey and Henrici (7) for specific references. Mention may be made, however, of some of the studies of Hansen (4) who found, in investigating Danish soils, that vineyard and orchard soils were more abundantly supplied with yeasts than other soils examined. Thus *Saccharomyces* were found in 67% of samples of soil under orchard trees, in 30% of samples under neighboring woods, and in but 19% of samples of more remote soils. Hansen believed that the majority of yeast species passed through a more or less definite cycle in nature, their most important breeding ground being the juice of fruits, with soil as their winter habitat from which they may be carried by wind, rain, or insects to reinfect the fruit and again multiply, particularly where they can come in contact with the sweet juice.

The most recent studies on soil yeasts appear to be those of Starkey and Henrici (7) and Ciferri (1). These investigators studied soil yeasts from a different point of view from that of the earlier researches, investigating yeasts as representatives of the soil flora and as possible active agents in soil microbiological processes. Starkey and Henrici found yeasts in small numbers in 39 of 87 samples of soil, noticing no correlation between the occurrence of yeasts and soil type, nature of crop or the season of the year. They were found in such small numbers and were so haphazard in distribution that the authors concluded that they play no part in soil transformation. The findings of Ciferri likewise tend to support this view.

In the works referred to, honey fermentation has not been considered, and since no data appear to be available regarding the sugar-tolerance of the types isolated, no previous information is at hand regarding the possible rôle of soil yeasts as active agents in honey fermentation.

Experimental Work

In the experiments here reported an examination of soil from six different locations on the Central Experimental Farm was continued throughout a twelve-month period, the sources being as follows:

1. Soil from flower garden.

2. Soil from Central Experimental Farm apiary, new ground being used for first time.
3. Soil from apiary, old ground.
4. Soil from orchard.
5. Soil from clover field.
6. Soil from cereal field.

Samples of soil were taken from a depth of 1 to 2 in., portions of approximately one gram being transferred aseptically to sterile tubes containing 80% honey broth of the following composition:

Honey	800 gm. per litre
Peptone	1.0 " " "
K_2HPO_4	1.0 " " "
$MgSO_4$	0.5 " " "
Ammonium tartrate	0.5 " " "
NaCl	0.1 " " "
$CaCl_2$	0.1 " " "

This medium was selected to permit of the development of sugar-tolerant types only. Our previously reported experiments had shown that 80% honey broth was a suitable medium for the elective cultivation of honey-fermenting types to the exclusion or ordinary non-osmophilic yeasts. It was prepared by mixing the ingredients named, the honey being previously warmed, and adding distilled water to a total volume of 1,000 cc. It was distributed in tubes in 10 cc. amounts and sterilized at 15 lb. pressure for 20 min. When ready for use it had a pH value of approximately 4.2.

From each location 12 tubes were inoculated with soil at each time of sampling as indicated in Table I and the cultures incubated at 20-25° C. to observe the development of fermentation, tubes being held if necessary for five weeks before finally recording negative results. At weekly intervals the tubes were agitated to insure better contact of the soil organisms with the medium. At the end of the five-week period transfers were made from all tubes showing evidence of fermentation to fresh tubes of 80% honey broth, for the purpose of confirming honey fermentation, particularly in such cases where slight evolution of gas might have originated from CO_2 evolved by soil bacteria within the soil inoculum. In these control tubes yeasts were the only organisms encountered, the bacterial flora of the soil being doubtless suppressed through the combined agencies of the high osmotic index and high hydrogen ion concentration of the medium selected. In Table I are recorded the inoculations made throughout the year and the findings observed.

TABLE I

INCIDENCE OF SUGAR-TOLERANT YEASTS IN SOIL

Date of sampling	Percentage of samples showing fermentation					
	1 Flower garden	2 Apiary—new ground	3 Apiary—old ground	4 Orchard	5 Clover field	6 Cereal field
May 22, 1929	0	8.3	66.7	0	0	0
June 5, 1929	0	16.7	83.3	0	8.3	0
June 18, 1929	0	16.7	50.0	0	0	0
July 31, 1929	0	16.7	50.0	0	0	0
Aug. 19, 1929	0	50.0	50.0	0	0	0
Aug. 30, 1929	0	25.0	66.7	0	0	0
Sept. 11, 1929	0	16.7	41.7	0	0	0
Sept. 25, 1929	0	0	75.0	0	0	0
Oct. 16, 1929	—	33.3	66.7	—	—	—
Nov. 21, 1929	—	58.3	41.7	—	—	—
Dec. 16, 1929	—	25.0	50.0	—	—	—
Jan. 16, 1930	—	25.0	58.3	—	—	—
Feb. 13, 1930	—	25.0	33.3	—	—	—
Mar. 18, 1930	—	33.3	75.0	—	—	—
Apr. 17, 1930	—	16.7	50.0	—	—	—

The results outlined in Table I indicate that only in the case of the apiary ground is the soil regularly infected by sugar-tolerant yeasts. Samples taken from the other locations from May until the end of September, when yeast infection of the soil might be considered most probable, all yielded negative results with the exception of a single portion out of the 12 taken on June 5 from the clover field. In this case fermentation was but slight, while subsequent attempts at cultivation of the organisms on concentrated honey media were not successful, thus casting doubt on the sugar-tolerance of the organisms concerned.

The findings support the view that ordinary soil is not to be regarded as a primary source of infection of honey-fermenting yeasts, nor are sugar-tolerant types to be considered as members of the normal soil microflora. On the contrary, there is reason to believe, from the data obtained, that when soil contains such yeasts it is infected under unusual circumstances, such as proximity to bee hives or other sources of saccharine solutions of high density. In the case of the apiary soil it is of interest to note that the soil from the older part of the apiary was much more heavily infected than soil being used for the first time. For the former, 57.2% of all tubes showed fermentation as compared with 24.4% for the latter. It appears reasonable to conclude that in the apiary, the soil becomes more or less rapidly contaminated with honey-fermenting yeasts from droppings of wax, nectar and from dead bees which are continually being removed from the hives. That hive nectar is regularly infected with such yeasts has been shown in studies at this laboratory, while Wilson and Marvin (8) report their isolation from the bodies of bees examined by them. While soil in the apiary, therefore, apparently serves as but a

resting place for honey-fermenting yeasts, presenting no favorable opportunity for growth or multiplication, yet it may constitute a source for seasonal reinfection through such agencies as wind, insects, etc.

It is of interest to note that honey-fermenting yeasts are able to remain viable in the soil under Canadian winter conditions. At the time of taking the samples from December to March the soil was in a frozen state, the average daily minimum temperatures for the months in question being respectively— −13.6°, −15.4°, −15.3° and −7.4° C. Sugar-tolerant yeasts, however, were found at every date examined. While observations made by us have shown them incapable of active growth below 10° C., yet they are to be regarded as cold-resistant organisms.

Yeast Types Isolated From Soil

From each tube showing fermentation isolations were made of the organisms concerned by plating out on an agar medium of 60% honey, similar to that used in former studies (6), incubation being at 28° C. In all cases yeasts alone were found to grow, and from well isolated colonies subcultures were made for replating and examination in pure culture. In most instances three platings were made on the 60% honey agar medium preparatory to the detailed morphological and physiological examination.

Altogether 166 cultures were isolated, studied and compared. A preliminary survey indicated that they represented but a comparatively small number of different types. For preliminary comparison inoculations were made from each on 50% honey agar slants, on 15% honey agar flasks for giant colony formation, and into dextrose, saccharose and maltose solutions prepared by adding the carbohydrate in 10% concentration to a basic solution of 0.5% yeast extract broth (Difco). The appearance on the media, and the fermentation of the sugars, together with the microscopic appearance of the cultures, served as a basis for comparison and preliminary elimination of many cultures as being of identical types.

Cultures remaining after the preliminary survey were all subjected to more detailed scrutiny according to the following scheme:

(a) Microscopic observations on various media.

(b) Growth on honey agar slants, containing 15% and 70% honey respectively.

(c) Growth in honey broths of 15% and 70% honey.

(d) Giant colony formation on 15% honey agar.

(e) Growth on carrot, potato, in milk, and in gelatine media containing 15% and 70% honey respectively.

(f) Fermentation tests with the following: arabinose, xylose, dextrose, levulose, mannose, galactose, saccharose, maltose, lactose, raffinose, dextrin, mannite, dulcite and salicin.

(g) Comparison of growth at 37° C. on honey agar of high and low concentration (70% and 15%).

As a result of the comparative morphological and physiological tests the original 166 cultures were finally reduced to seven types which were considered sufficiently distinct to warrant consideration as six species with one sub-species. Of the seven types, five were classified as *Zygosaccharomyces*, the others being regarded as species of *Torula* and *Mycotorula* respectively. Three of the yeasts proved to be similar to types found in fermented honey (2, 6). Four corresponded to, and a fifth was found to be closely related to, types previously isolated from flowers commonly visited by bees (6).

In Tables II and III are shown respectively the frequency of the occurrence of the yeast types with regard to location and the season of the year. In the tables, duplicate cultures of identical types occasionally isolated from the same original tube are not considered.

TABLE II
INCIDENCE OF YEAST TYPES IN APIARY SOIL ACCORDING TO LOCATION

Yeast type	Number of times isolated		
	New ground	Old ground	Total
Zygosaccharomyces			
S3B2	17	19	36
S2L1 (Var. S3B2)	2	5	7
S3B5	1	1	2
S3B6	6	35	41
S3B11	14	37	51
Torula			
S3C3	0	6	6
Mycotorula			
S3C7	3	4	7
Totals	43	107	150

TABLE III
INCIDENCE OF YEAST TYPES IN APIARY SOIL ACCORDING TO SEASON

Date	Yeast type						
	S3B2	S2L1	S3B5	S3B6	S3B11	S3C3	S3C7
May 22, 1929	0	0	0	8	0	1	0
June 5, 1929	3	0	1	5	4	1	1
June 18, 1929	1	0	0	1	3	1	4
July 31, 1929	4	1	0	1	1	0	0
Aug. 19, 1929	1	2	0	1	7	1	0
Aug. 30, 1929	5	0	0	0	5	0	1
Sept. 11, 1929	2	2	0	1	1	0	1
Sept. 25, 1929	1	0	0	5	2	0	0
Oct. 16, 1929	0	1	0	4	7	0	0
Nov. 21, 1929	5	1	0	1	5	0	0
Dec. 16, 1929	6	0	0	0	3	0	0
Jan. 16, 1930	3	0	0	2	5	0	0
Feb. 13, 1930	3	0	0	1	3	0	0
Mar. 18, 1930	2	0	0	9	2	0	0
Apr. 17, 1930	0	0	1	2	3	2	0
Totals	36	7	2	41	51	6	7

Description of Yeasts Isolated

Culture S3B2 (*Zygosaccharomyces* sp.) (See Plate I)

In the young cultures on honey agar 15%, cells are round to slightly ellipsoidal, with thick walls, occurring singly, in pairs, in short chains and in small or large groups of adherent cells. The majority of the cells range from 3 to 5 μ in diameter, while occasional extremes are found of 2μ and 6 μ. Much the same appearance is noted in young cultures on honey agar 70%, the cells being, if anything, rather less uniform in size. In old cultures (eight weeks) on potato, some "giant" round cells may be seen, 9-10 μ in diameter. Reproduced asexually by budding. Spore formation, resulting from copulation, may be observed on a variety of media, such as honey agar, carrot and potato. From one to four ascospores observed in the ascus, two and three being most commonly encountered. Spores round to slightly oval, usually 3.5 to 4.0 μ in length with variations between 3.0 and 4.5 μ.

Honey agar 15%.—Growth scanty to moderate, at first beaded along line of inoculation and noticeably raised; spreading slightly in older cultures, the whole having rough dotted surface; cream colored, becoming slightly darker in older cultures; dull with butyrous consistency; medium unchanged.

Honey agar 70%.—Moderate to abundant growth, filiform or finely dotted; light brown in color; only slightly raised; dull with butyrous consistency.

Honey broth 15%.—Weak fermentation with gas, liquid finally appearing fairly clear; abundant, finely flocculent sediment; slight cream colored surface ring growth.

Honey broth 70%.—Active alcoholic fermentation with gas production; moderate light brown surface ring.

Giant colony, honey agar 15%.—Growth slow, tending to pile up with comparatively little spreading; irregular in outline with rough surface; cream colored with dull lustre and butyrous consistency.

Carrot.—Growth slow and usually scanty; raised and beaded along line of inoculation; at first cream colored, becoming pale brown in older cultures, with tendency to become chalky as medium dries up; dull lustre with consistency soft at first, but becoming tough in old cultures.

Potato.—Growth usually scanty, finely dotted along line of inoculation; at first cream colored, later raised to form a dry chalky mass.

Milk.—No change after eight weeks.

Gelatine.—No liquefaction observed after eight weeks with 15% or 70% honey gelatine.

Growth at 37° C.—Grows on honey agar 70%; no growth on 15%.

Fermentations.—Dextrose and levulose are fermented with acid and gas. With some strains there may be feeble fermentation of mannose. No fermentation observed with arabinose, xylose, galactose, saccharose, maltose, lactose, raffinose, dextrin, mannite, salicin or dulcite.

This yeast is regarded as the same as culture N 4 described by Lochhead and Heron (6) and isolated from a variety of flowers visited by bees.

Culture S2L1 (*Zygosaccharomyces* sp.)

This culture, while showing certain differences, possesses many characteristics in common with culture S3B2 described above. On the whole the growth is rather more abundant, while the fermentation of mannose, in addition to dextrose and levulose, is more pronounced. Giant colonies on honey agar 15% are rather larger, less raised, and with a smoother surface. Otherwise the description given under Culture S3B2 will suffice and hence this is regarded as a variety of the latter.

Culture S3B5 (*Zygosaccharomyces richteri*) (See Plate I)

Young cultures on honey agar 15% show mostly ellipsoidal cells, a few being round, with some lengthened to give a more cylindrical appearance. Occur generally singly or in pairs. Majority of cells vary from 4.0 to 6.5 μ in length, though forms as long as 9 or 10 μ may be encountered. Width of majority, 2.5 to 4.0 μ, though round forms may attain a diameter of 6μ. On honey agar 70% appearance much the same, though there is more tendency to adhere in masses. Reproduces asexually by budding, and also by means of ascospores formed by isogamic copulation. This may be observed on such media as honey agar, carrot, potato and in the surface ring growth of honey broth. Generally two or three spores may be observed in the ascus, two being the most frequent number. Spores are round to very slightly oval, measuring 3 to 4 μ in diameter.

Honey agar 15%.—Growth abundant, filiform, cream colored, becoming somewhat darker with age, particularly in central portion; old cultures tend to become covered with dotted growths, giving a verrucose appearance; lustre dull, consistency butyrous.

Honey agar 70%.—Growth moderate to abundant, filiform, light brown in color, becoming darker with age; surface slightly rough and sometimes broken by gas evolution; edge somewhat lobate and slightly wrinkled; at first glistening but later becoming dull; butyrous consistency.

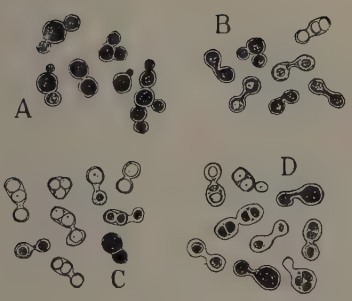
Honey broth 15%.—Active alcoholic fermentation with gas evolution; light brownish surface ring growth; abundant, coarsely flocculent sediment; liquid finally becoming clear.

Honey broth 70%.—Vigorous alcoholic fermentation with gas evolution; brownish surface ring growth.

Giant colony, honey agar 15%.—General form of colony round and slightly raised; at first cream colored, becoming light brownish with age; surface irregular, usually with concentric rings and faint radial markings; edge lobate; glistening at first but becoming dull; consistency butyrous.

Carrot.—Growth moderate to abundant, cream colored and slightly raised; edge irregular and lobate; surface contoured; at first slightly glistening but later becoming dull in lustre; consistency slimy but butyrous; medium unchanged.

Potato.—Growth rather scanty, cream colored; irregular in outline, raised with dull lustre; cheesy consistency; medium unchanged.



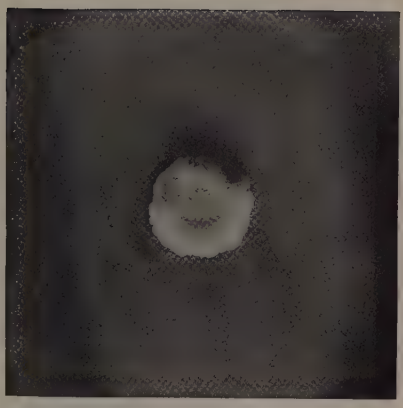
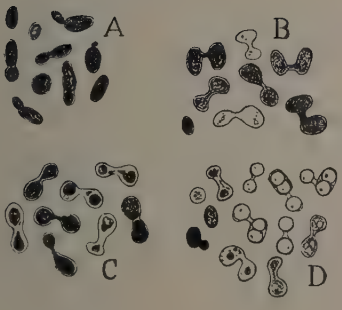


FIG. 1

CULTURE S3B2.

A. Young culture, honey agar 15%, three days, showing budding.
B. Eight-day culture, honey agar 15%, showing copulation.
C. Old culture, honey agar 70%, eight weeks, showing ascospores.
D. Eight-week culture on carrot, showing ascospore formation.
Photo. Giant colony, honey agar 15%, 48 days.



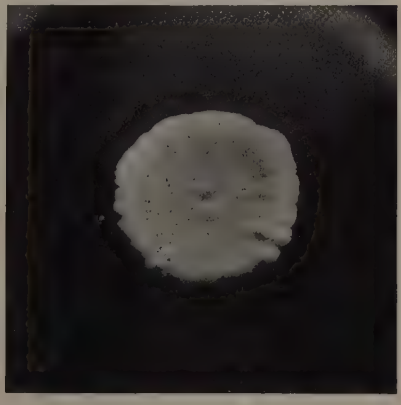


FIG. 2

CULTURE S3B5.

A. Young culture, honey agar 15%, two days, showing budding.
B. Four-day culture, honey agar 70%, showing copulation.
C. From surface ring growth, honey broth 15%, two months, showing copulation and early stages of spore formation.
D. Old culture, honey agar 70%, two months, showing ascospores.
Photo. Giant colony, honey agar 15%, 38 days.

Milk.—After eight weeks, no visible change in the medium.

Gelatine.—After eight weeks, liquefaction in honey gelatine 15%; none in 70%.

Growth at 37° C.—Growth on honey agar 70%; none on 15%.

Fermentation.—Dextrose, levulose, and mannose are fermented with acid and gas formation. No fermentation observed with arabinose, xylose, galactose, saccharose, maltose, lactose, raffinose, dextrin, mannite, dulcite or salicin.

This culture appears, from a comparison of cultural and morphological characteristics, to correspond closely to *Zygosaccharomyces richteri* isolated from fermented honey (6) and accordingly it is classed with that species.

Culture S3B6 (*Zygosaccharomyces nussbaumeri* var.) (See Plate II)

In young cultures on honey agar 15% cells ellipsoidal to round, being rather less elongated than in culture S3B5. Occur generally singly or in pairs with an occasional short chain or small group. Majority of cells 4 to 6μ in length with extreme variations between 2.5 and 8μ, very few being longer than 7μ. Width of majority ranges from 3 to 4 μ. On honey agar 70% the appearance is much the same. On carrot, and more particularly potato, multiple bud formation may be often seen from large, round cells 7 or 8 μ in diameter. Reproduces asexually by budding. Ascospores are formed as a result of copulation which appears to be heterogamic rather than isogamic, union between cells of unequal size being frequently observed. In addition, parthenogenesis appears to be frequent, in which ascospores may be formed without copulation. Sporulation may be readily observed on such media as carrot, potato, and old honey agar cultures. One to four spores may be present in the ascus, two or four being most frequently seen. They are slightly oval to round, generally 3 to 4 μ in length, the average size being 3.5 by 3.0 μ.

Honey agar 15%.—Growth abundant, at first filiform but later spreading somewhat; cream colored, becoming slightly darker with age; becoming raised with surface characteristically wrinkled and folded with much finer folds than culture S3B11; at first glistening but soon becoming dull; soft cheesy consistency; medium unchanged.

Honey agar 70%.—Growth abundant, filiform, light brown in color, becoming darker in older cultures; raised with rugose surface, although this characteristic is much less pronounced than on honey agar 15%; edge finely lobate and wrinkled; slightly glistening; butyrous consistency.

Honey broth 15%.—Active alcoholic fermentation with gas evolution; light brown surface ring growth; liquid finally becoming fairly clear; abundant, coarsely flocculent sediment.

Honey broth 70%.—Vigorous alcoholic fermentation with gas evolution; abundant brown surface ring growth, with tendency to pellicle formation.

Giant colony, honey agar 15%.—General shape of colony, round; tends to heap up into irregular folds and wrinkles, with much finer folds than culture

S3B11; cream colored; edge lobate and irregular in outline; at first glistening but later becoming dull.

Carrot.—Growth abundant, cream colored and raised; surface becoming finely wrinkled and folded; dull, with butyrous consistency; medium unchanged.

Potato.—Moderate growth, dull greyish white, somewhat spreading; slightly raised with irregular, finely verrucose surface, soft cheesy consistency; in older cultures tends to assume a chalky appearance as medium dries.

Milk.—After eight weeks no change observed in medium; finely dotted growth on side of tube above surface of liquid.

Gelatine.—Liquefaction of honey gelatine 15%, none observed in 70% after eight weeks.

Growth at 37° C.—Grows well on honey agar 70%, much less vigorously on 15%.

Fermentation.—Dextrose, levulose, mannose, saccharose and maltose are fermented with acid and gas. No fermentation observed with arabinose, xylose, galactose, lactose, raffinose, dextrin, mannite, dulcite and salicin.

This yeast has much the same cultural appearance and physiological characteristics as *Zygosaccharomyces nussbaumeri* (6), isolated from fermented honey and also found in the nectar of a number of flowers. Some apparent differences, however, have been noted, but we are inclined to regard the culture, at least tentatively, as a variety of *Z. nussbaumeri.*

Culture S3B11 (*Zygosaccharomyces* sp.) (See Plate II)

In young cultures on honey agar 15%, cells are ellipsoidal to round, generally occurring singly, occasionally in pairs or small groups. The great majority of the cells measure 4 to 6 μ in length, and are fairly uniform in size, very few exceeding these limits. Width of majority, 3 to 4 μ. On honey agar 70%, the cells are very slightly smaller. Reproduces asexually by budding. Ascospore formation, resulting from isogamic copulation, may be observed on such media as honey agar, carrot and potato, while, in addition, parthenogenesis appears to be fairly common, particularly on potato. Generally one to four spores in the ascus, two and three being the most frequent numbers encountered. Spores slightly oval to round, 3 to 4 μ in length, the average size being 3.5 to 3.0 μ.

Honey agar 15%.—Growth abundant, filiform, then spreading slightly; at first cream colored, becoming somewhat darker with age; becomes characteristically raised and wrinkled, being more coarsely folded than culture S3B6; at first glistening, but later becoming dull; consistency slightly dry and cheesy; medium unchanged.

Honey agar 70%.—Growth abundant, filiform, light brown in color, becoming darker with age; slightly raised with rugose surface though not as characteristically so as on honey agar 15%; edge lobate; lustre rather dull; consistency butyrous.

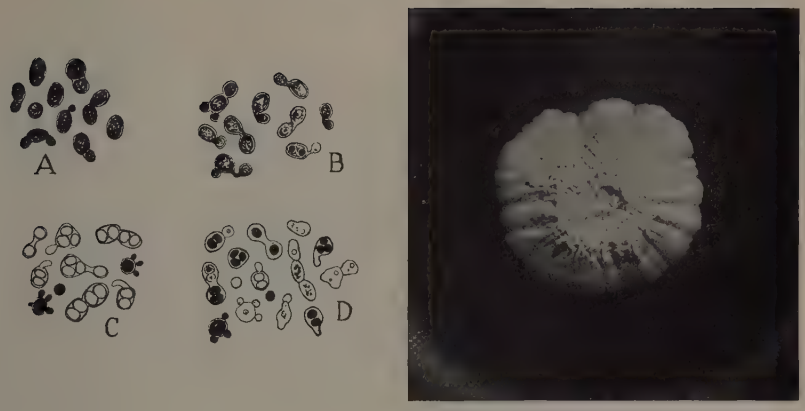


FIG. 3

CULTURE S3B6.

A. Young culture, honey agar 15%, two days.
B. Eight-day culture, honey agar 15%, showing copulation of apparently heterogamic type
C. Carrot culture, nine weeks, showing ascospores.
D. Five-month old culture, honey agar 15%.
Photo. Giant colony, honey agar 15%, 38 days.

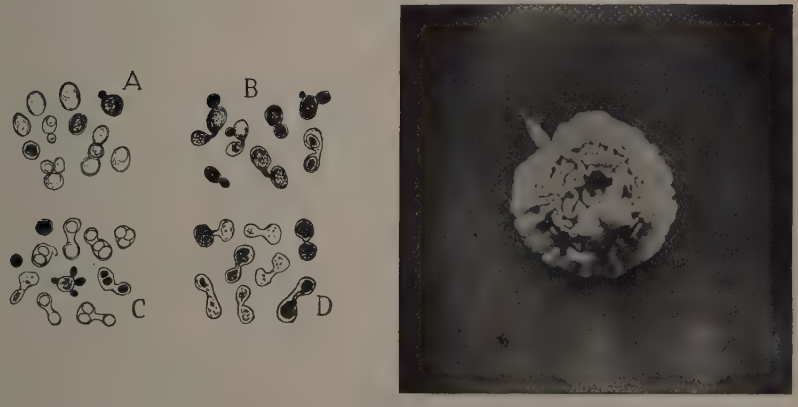


FIG. 4

CULTURE S3B11

A. Young culture, honey agar 15%, two days, showing budding.
B. Twelve-day culture on carrot, showing copulation.
C. Old culture on potato, nine weeks, showing ascospores.
D. From eight-week culture on potato, showing copulation.
Photo. Giant colony, honey agar 15%, 102 days.

Honey broth 15%.—Active alcoholic fermentation with gas production; moderate, light brown surface ring; abundant, flocculent sediment; liquid remaining turbid.

Honey broth 70%.—Vigorous alcoholic fermentation with gas evolution; abundant brownish surface ring growth with tendency to pellicle formation.

Giant colony, honey agar 15%.—General form of colony, round; becomes heaped up into irregular folds, giving a very wrinkled appearance, with folds of coarser type than with culture S3B6; cream colored, becoming darker with age; edge lobate, lustre becomes dull.

Carrot.—Growth abundant, cream colored, somewhat spreading and irregular in outline; raised with rough surface which may show folds; butyrous consistency; medium unchanged.

Potato.—Growth moderate, cream-colored and spreading somewhat; surface irregular; at first glistening but soon becoming dull; consistency butyrous; medium unchanged.

Milk.—Finely dotted growth on side of tube above surface of liquid; no change observed in the medium after eight weeks.

Gelatine.—Slow liquefaction in honey gelatine 15% noticeable after 10 weeks; none observed in 70%.

Growth at 37° C.—Grows well on honey agar 70%, less abundantly on 15%.

Fermentations.—Dextrose, levulose, mannose, saccharose, and maltose are fermented. No fermentation observed with arabinose, xylose, galactose, lactose, raffinose, dextrin, mannite, dulcite or salicin.

Although this culture has the same fermentative properties as culture S3B6, and shows certain physiological similarity with the latter, yet apparent cultural and morphological differences incline us to view it as a separate type.

Culture S3C3 (*Torula* sp.) (See Plate III)

Young cultures on honey agar 15% show predominantly round cells, though occasionally slightly ellipsoidal forms may be observed. Cells occur usually in pairs consisting of a mother cell with bud, although sometimes multiple bud formation is seen. Cells vary from 2.0 to 4.5 μ in diameter, the majority having a diameter of 2.5 to 3.0 μ. On honey agar 70% the appearance in young cultures is much the same, the cells being, if anything, very slightly smaller. Reproduces asexually by budding. Spore formation has not been observed.

Honey agar 15%.—Growth abundant, filiform, cream to yellowish brown in color; only slightly raised, with smooth surface slightly bevelled towards edge; edge faintly lobate; in older cultures the flat, central portion shows finely dotted development and faint markings giving etched appearance; glistening, though less so in old cultures; soft, butyrous consistency; medium unchanged.

Honey agar 70%.—Growth abundant, filiform, light brown in color, becoming slightly darker with age; very slightly raised, with bevelled edge which is lobate; surface smooth and dull; consistency butyrous, becoming slightly viscous in old cultures.

Honey broth 15%.—Active alcoholic fermentation with gas evolution; moderate, cream colored surface ring; abundant, finely flocculent sediment; liquid remains cloudy with finely divided suspension.

Honey broth 70%.—Active alcoholic fermentation with gas evolution; moderate light brown ring growth at surface of liquid.

Giant colony, honey agar 15%.—Round, rather flat colony with finely lobate edge, dark cream colored; surface smooth at first, but becoming covered in central portion, a few millimetres from edge, with secondary dotted outgrowths of varying size giving a mottled, verrucose appearance; slightly glistening, with soft, butyrous consistency.

Carrot.—Growth fairly abundant, filiform and raised; dark cream colored and somewhat glistening; surface slightly verrucose; consistency butyrous; medium unchanged.

Potato.—Moderate growth, cream colored and dull; somewhat raised in centre with contoured surface; cheesy consistency; medium unchanged.

Milk.—Finely dotted growth on side of tube above surface of liquid; no change observed in medium after eight weeks.

Gelatine—No liquefaction observed after eight weeks in honey gelatine; after three months, slight liquefaction in the 15%, none in the 70% medium.

Growth at 37° C.—Grows well on honey agar 15%; much less vigorously on 70%.

Fermentations.—Dextrose, levulose, mannose, saccharose and raffinose are fermented. No fermentation observed with arabinose, xylose, galactose, maltose, lactose, dextrin, mannite, dulcite and salicin.

This yeast is closely related to two types of *Torula* isolated by Lochhead and Heron (6) from the nectar of a number of flowers.

Culture S3C7 (*Torula* (*Mycotorula*) *mellis*) (See Plate III)

In young cultures on honey agar 15%, cells are mostly oval in shape, though spherical, lemon-shaped and cylindrical forms may be noted, there being considerable variation, both in form and size. Cells occur singly, in pairs and in short chains. In addition, there is a tendency, even in young cultures, for cells to germinate into promycelia of varying length. Cells are notably thick-walled. Oval cells vary in length from 4 to 9 μ and in width from 2.5 to 6.0 μ, the average size being 4.5 by 6.5 μ. The more elongated, cylindrical cells may attain a length of 10 to 20 μ, often joined to form chains of mycelium-like growth. In young cultures on honey agar 70%, much the same type of growth is observed, the elongated cells being relatively more numerous and with more tendency to form mycelia. Older cultures on this medium show round, oval, or cylindrical cells containing highly refractile, round bodies which are shown to be oil or fat globules. On carrot, old cultures show very thick-walled, spherical cells often joined in chains or groups of varying size. Reproduces asexually by budding. Spore formation has not ben observed on any of the media employed.

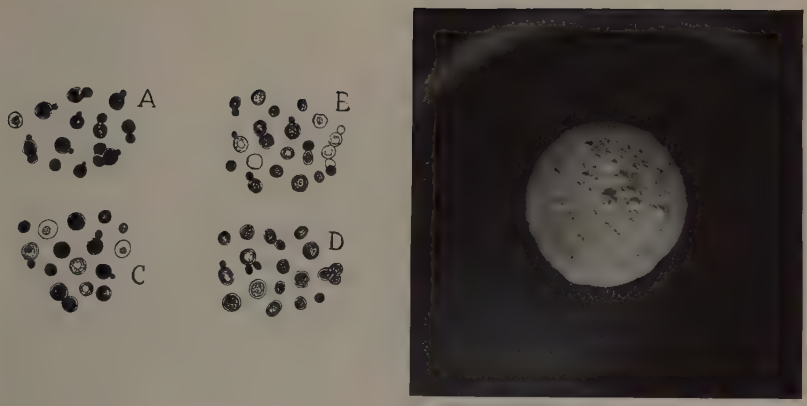


FIG. 5

CULTURE S3C3.

A. Young culture, honey agar 15%, two days, showing budding.
B. Old culture, honey agar 15%, nine weeks.
C. Eight-week culture on honey agar 70%.
D. Four-week culture on carrot.
Photo. Giant colony, honey agar 15%, 38 days.

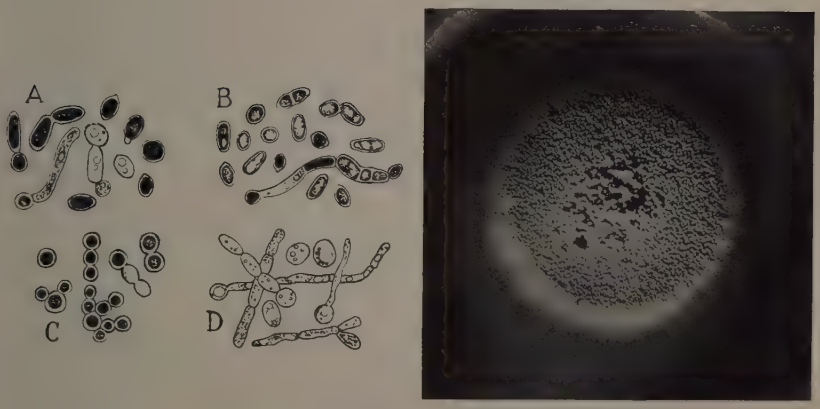


FIG. 6

CULTURE S3C7.

A. Young culture, honey agar 15%, two days, showing thick-walled vegetative cells, also cells germinating to form promycelia.
B. Seven-day culture, honey agar 70%, showing variety of cell forms.
C. From carrot culture, nine weeks, showing thick-walled, round cells, with budding and chain formation.
D. From surface film growth, honey broth 70%, eight weeks, showing tendency to mycelial growth.
Photo. Giant colony, honey agar 15%, 38 days.

Honey agar 15%.—Growth abundant, at first filiform, raised and finely wrinkled, dirty white in color with dull lustre; as culture ages color changes to yellowish, brownish and greenish, and as growth increases, it becomes continually darker, showing a dark greenish-brown shade which may become almost black. The surface becomes folded and wrinkled, with irregular globular outgrowths; finally dirty whitish to grey fuzzy outgrowths appear in patches on the surface, giving a fungoid appearance; consistency variable, dry cheesy to tough; medium shows a greenish-brown discoloration.

Honey agar 70%.—Growth abundant, spreading somewhat; greenish-brown in color, surface finely wrinkled with dry appearance; in old cultures growth becomes dark brown; rather dry, flaky consistency.

Honey broth 15%.—Moderate alcoholic fermentation; abundant greenish yellow surface growth which may sink; after two months liquid almost clear with abundant, flocculent, brownish yellow deposit.

Honey broth 70%.—Vigorous alcoholic fermentation with gas evolution; yellowish growth on surface of liquid, with ring growth on side of tube.

Giant colony, honey agar 15%.—General form of colony, round; spreading with edge rather indefinite owing to growth below the surface of the medium; at first brownish-yellow; surface raised, more especially towards centre, becoming covered with small globular outcroppings; as culture ages the color darkens, becoming brownish and passing through greenish shades to almost black; finally dirty grey or white fuzzy patches appear.

Carrot.—Moderate growth, at first pale yellowish in color, irregular, raised and lumpy, with dry appearance; becomes darker with age as it passes through greenish brown and finally becomes black; dull in lustre, with dry, flaky consistency; medium shows dark discoloration.

Potato.—Growth moderate to abundant, irregular, raised and folded, with dry appearance; at first light colored, becoming darker with age to a very dark brown; dull in lustre with dry flaky consistency.

Milk.—Dark greenish to brownish growth above surface of liquid on side of tube; medium shows a slight brownish discoloration; soft curdling after eight weeks without any marked change in reaction.

Gelatine.—Liquefaction in honey gelatine 15%, none observed in 70%.

Growth at 37° C.—The organism grows on honey agar 70%, though no growth was obtained on 15%.

Fermentations.—Dextrose, levulose, mannose, galactose, and maltose are fermented with acid and gas. No fermentation observed with arabinose, xylose, saccharose, lactose, raffinose, dextrin, mannite, dulcite or salicin.

This culture is regarded as being identical with *Torula mellis*, isolated by Fabian and Quinet (2) from fermented honey. It was also isolated by Lochhead and Heron (6) from a large number of flowers visited by bees, being in fact the most frequently encountered sugar-tolerant organism from this source. Its apparent relationship to *Mycoderma* together with its undoubted capacity for carbohydrate fermentation inclined the last named authors to regard it as a species of *Mycotorula*.

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STUDIES ON LIGNIN AND RELATED COMPOUNDS
III. GLYCEROL-CHLOROHYDRIN-LIGNIN[1]

By Harold Hibbert[2] and John Bernard Phillips[3]

Abstract

Glycerol α-monochlorohydrin has been found to be an effective extraction agent for the removal of lignin from spruce wood meal. Previous evidence for the view that a compound is formed between the lignin and the extraction medium is supported by the results of methoxyl and halogen analysis, and those of hydrolysis.

In two previous communications (7, 8) it has been shown that lignin can be readily extracted from spruce meal by the aid of glycol, or glycol monomethyl ether, using either iodine or preferably traces of hydrogen chloride as catalyst.

From the evidence obtained, and the mild operating conditions used, it seemed reasonable to assume that no deep-seated changes in the constitution of the lignin, as present in the wood, had actually occurred.

These experimental results indicated that some kind of union between the glycol, or glycol methyl ether, respectively, and the lignin, had taken place, the nature of which, however, remained unsolved.

In the present research, other hydroxylic solvents such as glycerol and lactic acid, were employed, and found to act in the same way. The conditions for the reaction in each case were also investigated.

The difficulty in the detection of glycol or glycol methyl ether in the material removed by hydrolysis of glycol-lignin or glycol-ether-lignin respectively, prompted the thought that the use of a halogen-hydroxy derivative in place of the glycol might give valuable information from the point of view of indicating a definite union of the solvent and the lignin complex. Glycerol α-monochlorohydrin was therefore selected as an extraction medium. The conditions for the reaction were found to be essentially different than when glycol, glycerol, etc., were used.

Extractions were made both with and without the addition of hydrogen chloride as catalyst. The chlorohydrin used was found by titration with 0.1 *N.* caustic soda to have an acid value equivalent to 0.176% free HCl; at the end of the extraction the mixture usually contained about three times this amount of acid, so that a certain amount of free acid was evidently supplied by the chlorohydrin itself. The pronounced reactive character of this solvent made the isolation process much more difficult than in the case of glycol or glycerol. After a few hours' treatment, the mixture was converted into a very viscous mass, this making the separation of the extract by filtration an extremely tedious process. The glycerol chlorohydrin-lignin obtained by precipitation with water was colloidal, and very difficult to free from adsorbed

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Contribution from the Industrial and Cellulose Chemistry Laboratories, McGill University, Montreal, Canada. Constructed from the thesis presented by J. B. Phillips to the Graduate School of McGill University, in partial fulfilment of the requirements for the degree of Master of Science.

[2] *Professor of Industrial and Cellulose Chemistry, McGill University.*
[3] *Post-graduate Student, McGill University.*

chlorine. There was also danger of decomposition of the product, or loss of chlorine by hydrolysis, if the washing with water were continued too far. The dried substance was amorphous, and light brown in color, very similar to the products obtained by the use of glycol, glycerol, lactic acid, etc. It was quite soluble in dilute alkali, and could be reprecipitated by the addition of acid. Considerable decomposition of the substance could be effected by hydrolysis in successive stages, and the product left was insoluble in alkali.

The analytical results for this lignin-chlorohydrin product indicate that a union of some kind has taken place between the solvent and the lignin and the presence of chlorine in the product in definite amount supports this view. The presence of reducing substances in the filtrates after hydrolysis could not be detected by Fehling's solution.

The methoxyl content of the glycerol-chlorohydrin-lignin was found to be 15.6%, compared with 18.5% for glycol-lignin (7). A comparison of these results provides an indirect method for estimating, approximately, the molecular weight of the glycerol-chlorohydrin-lignin building unit. If it be assumed that the action of the two solvents on the lignin contained in the spruce meal is identical, then the two should differ only by the difference in structure of the solvents used, namely: lignin—$CH_2OH.CH_2OH$ and lignin—$CH_2OH.CHOH.CH_2Cl$, in other words, by the value $-CHCl$, thus representing a weight of 48.5 with reference to the molecular weight of the lignin derivative.

There should be the same number of methoxyl groups in each of these two substances, that is, the same absolute weight of the OCH_3 radicles, although the percentages would differ. If M_c represents the molecular weight of the glycerol-chlorohydrin-lignin, M_g that of the glycol-lignin and x the actual weight in grams of methoxyl radicle (the same in each product), then:

$$x/M_c = 0.156 \text{ and } x/M_g = 0.185;$$
$$x = 0.156M_c = 0.185M_g;$$
$$M_c = 185M_g/156 = 1.186M_g;$$
$$= 1.186(M_c - 48.5);$$
$$= 309.$$

Molecular weight determinations by the cryoscopic method were attempted but no satisfactory solvent could be found. A sharp melting point could not be obtained with pinacone, which dissolved the substance completely. Acetophenone also was found to dissolve the product, but the irregularities in the observed freezing points indicated a large degree of association. The first readings, however, were recorded, and the calculated molecular weights plotted against concentration in the usual way, and the curve extrapolated to zero concentration, giving a value of 350 for molecular weight. This is of the same order as the value 309 by the other method. The value by this method is probably more accurate than the one calculated from the methoxyl percentages.

If the chlorohydrin residue were split off from a molecule of weight about 350, this would correspond with a loss of approximately 31%, while successive

hydrolyses with 8% HCl have been found to remove 24.48%; lignin determinations made by hydrolysing with 75% H_2SO_4 showed 25.8% non-lignin material, which agrees with the above.

The residue after hydrolysis with 8% HCl was insoluble in dilute caustic soda, probably due to the loss of the chlorohydrin residue, and to a more or less deep-seated decomposition in the molecule.

In a compound with a building unit of 350, one methoxyl group in the unit would correspond with 8.8% approximately, so the result obtained, 15.6%, is approximately equivalent to two OCH_3 groups. After methylation the methoxyl value, 23.2%, indicates that one hydroxyl group has been methylated.

The percentage of chlorine (5.55%) was confirmed by analysis of the products of different extractions. This value is too low for one atom of chlorine in a unit of 350 molecular weight, and it is possible that chlorine is split off very easily in this product, by hydrolysis, in the lengthy washing process. The percentage of chlorine corresponds very well with one atom of the halogen to two lignin residues of approximately the above weight.

It is apparent that the chlorine value obtained is not easily reconcilable with the results found from the methoxyl determinations.

The results in any event are only to be regarded as in the nature of a preliminary survey but do provide definite supporting evidence that in the extraction with glycerol α-monochlorohydrin a union between the lignin and the solvent takes place.

Experimental

Preparation of glycerol-chlorohydrin-lignin

The preparation of the spruce meal, extraction with the solvent, treatment of the residue and the recovered lignin product, were carried out by the methods already described in previous communications (7, 8). In the case of the glycerol chlorohydrin, however, filtration of the wood meal residue from the extract was extremely difficult. This was carried out most satisfactorily by using a Büchner funnel surrounded by a hot water jacket. Dilution of the viscous mass with acetone was found to be of assistance, but sometimes caused precipitation of flocculent solid matter from the liquid extract; also much of the acetone was lost due to its volatility.

The washing of the glycerol-chlorohydrin-lignin product was shortened by centrifuging the material after washing by decantation with water two or three times. The material which remained in suspension was coagulated by heating the decanted liquid to 60° C. for a few minutes, with the addition of a few cc. of dilute sodium sulphate solution, followed by filtration.

The material was washed until free of adsorbed chlorine impurities, dried at 65° C. in vacuo, and then over phosphorus pentoxide, leaving a pale brown, amorphous powder.

TABLE I

EXTRACTIONS OF SPRUCE MEAL WITH VARIOUS SOLVENTS

Solvent	Bone dry wt. of spruce meal* in gm.	Wt. of solvent in gm.	Grams HCl per 100 gm. spruce meal	Loss in weight, per cent.	Lignin recovered per 100 gm. of wood	Time of extraction in hr.	Temperature, in deg. C.
Glycol	124.6	1532	0.49	21.8	4.6	6	100
Glycerol	11.0	110	0.50	1.1	0.23	6	100–105
Glycerol	10.9	109	1.00	39.3	4.14	3	125
Glycerol	10.0	100	1.00	28.0	3.0	6	100–110
Glycerol-chlorohydrin	13.8	133	none	—	—	6	100
Glycerol-chlorohydrin	2.0	20	none	2.5	0.5	6	50–55
Glycerol-chlorohydrin	2.9	32	none	20.6	3.5	6	75
Glycerol-chlorohydrin	25.0	200	0.50	49.2	10.1	6	80
Glycerol-chlorohydrin	25.0	200	0.20	38.9	15.2	6	80
Lactic acid	10.0	120	0.50	42.0	14.1	10	100

**Previously extracted with equal parts of 95% alcohol and benzene, and with water.*

Glycerol-chlorohydrin-lignin was completely soluble in dilute caustic alkalis, in phenol, $\beta\beta'$-dimethylpropylene glycol, pinacone and acetophenone; slightly, in concentrated alkalis, acetone, 95% alcohol and dilute sodium carbonate. It was insoluble in water, absolute alcohol, benzene and ethyl ether. By the method of Tollens (5) the pentosans content was found to be 1.00 and 1.38%. This was probably not attributable to pentosans but to formaldehyde (1, 7) removed in the hydrolysis. Analysis: (Carius (2) method) samples: 0.3230, 0.2761, 0.2283, 0.3682, gm.; AgCl: 0.0700, 0.0640, 0.0510, 0.0829 gm.; Cl: 5.36, 5.73, 5.52, 5.57%. Mean value 5.55%. Lignin content (Klason's (9) method), samples: 0.0710, 0.1280 gm.; residues: 0.0530, 0.0945 gm.; lignin: 74.6, 73.8%. Methoxyl: samples: 0.2586, 0.2250 gm.; AgI: 0.3060, 0.2650 gm.; OCH_3: 15.64, 15.55%. Mol. wt. sample, 0.0295, 0.0634, 0.0784, 0.1386 gm.; solvent acetophenone, 12.2, 13.4, 13.0, 12.4 gm.; $\triangle$, 0.030, 0.051, 0.057, 0.008°C.; mol. wt. 457, 523, 596, 788 (extrapolated value, 350).

Hydrolysis of glycerol-chlorohydrin-lignin

Glycerol-chlorohydrin-lignin was hydrolysed by boiling with 8% hydrochloric acid for one hour at a time, in successive stages (3). The results of one typical series of four hydrolyses are given here, the figures representing the percentage loss based on the original weight.

1st hydrolysis	14.35% loss
2nd hydrolysis	5.35
3rd hydrolysis	3.48
4th hydrolysis	1.30
Total	24.48%

Lignin content of residue determined by Klason's method (9), samples: 0.3545, 0.2375 gm.; residue: 0.3324, 0.2185 gm.; lignin, 93.8, 92.0%.

Methylation of glycerol-chlorohydrin-lignin

The methylation was carried out by the agency of dimethyl sulphate and potassium hydroxide (6), three successive treatments being sufficient to complete the reaction (7, 11). Analysis: (Perkin (10) method) samples: 0.2684, 0.2973 gm.; AgI: 0.4740, 0.5205 gm.; OCH_3: 23.31, 23.10%.

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A NEW ADIABATIC CALORIMETER[1]

By W. H. Barnes[2] and O. Maass[3]

Abstract

A new adiabatic calorimeter equipped with a radiation thermel is described. Measurements of the heat capacity of ice over various temperature ranges are presented in order to illustrate the method of handling the calorimeter and to find the order of accuracy of this piece of apparatus. The latent heats of fusion of ice at 0°C. and at − 3.0°C. are found.

The novel feature of the new calorimeter is shown to be the radiation thermel for indicating any difference between the temperatures of the inner calorimeter and the outer bath. This instrument consists of a multiple junction thermocouple of which one set of junctions receives heat from the inner calorimeter vessel by radiation. The accuracy of the calorimeter is shown to depend on the magnitude of the temperature drop in the inner vessel and on the method for reading the temperatures involved. In the experiments with ice a probable accuracy of about 0.2% is obtained with temperature drops of the order of 2°, measurement of which is made with a Beckmann thermometer.

Introduction

A few years ago the authors of this paper published the results of some heat capacity measurements made with an adiabatic calorimeter (9). Shortly afterwards Lipsett, Johnson and Maass (8) described a special form of adiabatic calorimeter for determinations of heats of solution. One of the novel features of this piece of apparatus was the use of a radiation thermel for indicating any difference in temperature between the inner vessel and the outer bath of the calorimeter. The present paper is concerned with the use of a radiation thermel in the more general form of adiabatic calorimeter described by Maass and Waldbauer (10) and by Maass and Barnes (9).

In its original form the calorimeter depended on a multiple junction thermocouple for indications of any difference in temperature between the outer and inner baths. This thermocouple, in the form of an inverted U, was mounted with one arm in the outer bath and the other passing through the cover of the calorimeter into the water in the inner vessel. This arrangement had three obvious disadvantages. In the first place contact was made between the inner liquid and the outside air through the thermocouple arm; secondly, an uncertain water equivalent was introduced into the calculations, and finally, the thermocouple arm filled valuable space inside the calorimeter proper. In spite of these factors, however, the calorimeter gave results with an accuracy of within 0.5%. With the outer bath kept at exactly the same temperature as the inner calorimeter the accuracy of the results should depend on the accuracy with which the temperature of the outer bath is read. In the experiments referred to (9), the temperature drop varied from about 0.2° to 2.0° depending on the initial temperature of the substance whose heat

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Contribution from the Physical Chemistry Laboratory, McGill University, Montreal. The research described in this paper was undertaken during the session 1926-27.

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capacity was to be measured. The temperature of the outer bath was found by means of a calibrated Beckmann thermometer. If about .001 be allowed as the probable error in reading the Beckmann thermometer, then the accuracy of the heat measurements should have varied from about 1% to 0.1%, because each measurement involved two readings of the thermometer. The fact that the accuracy attained was not so high as the lower limit, indicated that an improvement might be effected in keeping the outer bath more nearly at the temperature of the inner calorimeter. This involved some change in the thermocouple and the radiation thermel was developed for this purpose.

Description of New Adiabatic Calorimeter

Reference should be made to the original papers (9, 10) for the details of the early calorimeter. For the sake of brevity a complete description of this piece of apparatus is not repeated here.

In order to increase the sensitivity of the old calorimeter the original multiple thermocouple consisting of two sets of junctions of copper and constantan was replaced by a radiation thermel. This instrument consisted of eight pairs of junctions arranged in the form of a helix around the inner calorimeter vessel. Eight junctions made thermal contact with the inside wall of the outer jacket while the others were supported in air at a distance of about 3 mm. from the side of the inner calorimeter vessel, receiving heat by radiation.

The first thermel was mounted with this second set of junctions passing over ebonite collars for strength. A series of tests, however, showed that this arrangement was not sufficiently sensitive, due to the heat capacity of the ebonite.

In the final arrangement these junctions were mounted on small ebonite forks in such a way that the junctions were in effect suspended in air. (Fig. 1).

Copper and constantan wires were employed in making the thermel, the former of 0.20 mm. diameter and the latter 0.11 mm. diameter. Before soldering, the copper at the junctions was rolled to a thickness of 0.025 mm. and the constantan to 0.035 mm. so that after soldering each junction had a thickness of about 0.07 mm. The junctions were each about 1 mm. in length and were mounted on the small ebonite forks so that the wires were supported about 1 mm. from each side of the junctions. The flat surface of the junctions was placed tangentially with respect to the cylindrical wall of the inner vessel and these surfaces were blackened in order to make absorption of heat a maximum. The junctions in thermal contact with the inside surface of the outer calorimeter jacket were duplicates of the set already described. They were shellacked to very thin strips of mica which, in turn, were shellacked to the surface of the outer jacket. The connecting wires between pairs of junctions passed through air.

With this arrangement a deflection of 0.1 in. from zero on the galvanometer scale corresponded to a temperature difference of about 0.0007° between the inner calorimeter and the outer bath. With the original multiple junction

thermocouple the same deflection corresponded to a difference of about 0.0016°. The radiation thermel increased the sensitivity about twice.

Since a deflection of 0.02 in. could easily be detected, a difference in temperature of as low as 0.00014° between the inner calorimeter and the outer bath was quite apparent when the radiation thermel was employed.

The leads from the thermel passed to a Leeds-Northrup D'Arsonval galvanometer through a short-circuit switch and a contact switch. Both switches were of the clothes-pin anti-thermo-electric type (13) and were mounted on small bakelite panels. The wires were suspended in air as much as possible and, where necessary, were supported on porcelain insulators.

It was found necessary to enclose the galvanometer in the following system of insulation. A copper-lined wooden box was enclosed in a second box with an air space between the two. The latter was in turn surrounded by wool and contained in a third wooden box.

The leads from the thermel entered the inside box through glass tubes heavily coated with paraffin on the outside and inside.

These precautions were found to be necessary in order to minimize the effect of stray electrical currents on the galvanometer and to eliminate chance effects of unequal temperatures at the galvanometer binding posts.

The copper lining of the inner box was connected by a wire to a screw clamp which fastened to the cover of the outer jacket of the calorimeter and thus protected galvanometer and thermel with an equipotential shield (12). The padding of wool served as heat insulation and as a protection against mechanical vibration.

The galvanometer spot was obtained by focussing the filament of a large electric lamp on a scale by means of a double convex lens. The length of optical path from galvanometer mirror to scale was about 3.75 metres.

Stray electrical currents reaching the galvanometer were a source of considerable trouble but were eliminated completely by the precautions already described, in addition to connecting the casings of all motors and all metal parts of the apparatus to earth.

A series of tests made with the radiation thermel and the old open calorimeter showed a difference of about 0.02° between the temperature of the outer bath and that of the inner calorimeter when the galvanometer spot was kept on the zero mark. This difference showed a tendency to decrease after an arranged sudden drop in the temperature of the inner calorimeter. This effect was found to be due to the condensation of water vapor on the thermocouple junctions receiving their heat from the inner calorimeter. Condensation had the effect of warming these junctions and consequently the outer bath had to be maintained at a higher temperature than that of the inner calorimeter in order that no deflection of the galvanometer spot from zero should occur. At a lower temperature condensation was lessened and the effect decreased.

In order to prevent this occurrence a copper cover was constructed to fit snugly over the top of the inner calorimeter. This cover was fitted with light

ebonite chimneys which passed through brass tubes in the cover of the outer jacket for the passage of the wires to the stirrer and for the introduction of substances to be investigated. By means of vaseline the cover was made vapor tight and by having the ebonite chimneys in contact with the tubes in the outer cover, the inside surfaces of which also were covered with vaseline, the space inside the calorimeter jacket could be closed off completely from the access of water vapor either from the inner calorimeter or from the outside air.

By this means the difference in temperature between the inner and outer baths, when the galvanometer spot was maintained on the open-circuit zero, was reduced to 0.002° as determined with Beckmann thermometers. The final difference was found to be due, in spite of the insulation, to unequal temperatures of the two binding posts of the galvanometer due to the presence of hot water pipes on one side of the room. Further tests showed this difference to be very constant and also that no deflection was caused by the wires or switches in the galvanometer circuit. Consequently the position of the galvanometer spot when short circuited was taken as the true zero.

The closed calorimeter and the arrangement of the radiation thermel as finally adopted are shown (not drawn to scale) in Fig. 1. The outer bath, cover of calorimeter jacket, and inner stirrer have been omitted from this figure because they are described in detail in the previous paper.

In Fig. 1, L represents the outer jacket with the cover removed. M is the inner calorimeter vessel. I, I are the ebonite chimneys through which pass the wires to the inner stirrer. J is the ebonite chimney through which the substance under investigation is introduced. These three chimneys were made as thin as possible and were screwed into the copper cover K.

The elevation view in Fig. 1 is a section taken through the line marked XX in the plan. Consequently of the eight pairs of thermo junctions (A, B,

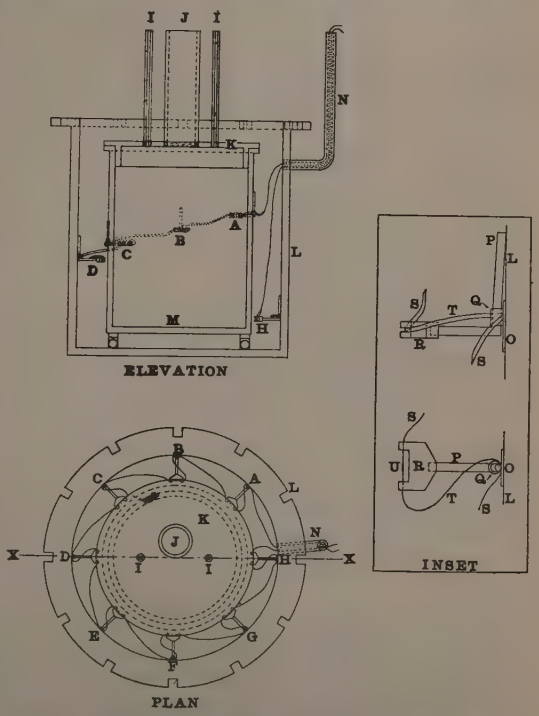

FIG. 1. *Side and plan views of the calorimeter, an enlarged view of one of the thermocouples.*

C, D, E, F, G, H) only five (A, B, C, D, H) appear in the elevation, and of these D and H are shown in cross-section. The wires to the galvanometer were of very small gauge copper and were double silk covered. They were soldered to the copper thermojunction wire near the junctions A and H and were carried out of the calorimeter system through the brass tube N. It should be noted that all the thermoelements are in series and are arranged symmetrically around the inner calorimeter vessel.

In the inset (Fig. 1) one of the thermocouples, with its support, has been drawn to a large scale. P is a light copper wire of sufficient strength to carry safely the ebonite fork R. Copper wires are marked with an S and constantan with a T. One junction U is placed between the prongs of the fork, as already described, while the other passes between the light ebonite collar Q and the thin strip of mica O. The upper end of the support P is soldered to the inner wall of the outer jacket L. The ebonite collar Q and the mica plate O serve to insulate electrically the junction between them from the support P and the wall of the outer jacket L. The mica strip O is so thin that no appreciable lag in the conduction of heat between the junction and the outer bath can occur. The junction U is so small compared with the curvature of the inner calorimeter vessel M that it is essentially parallel to it and the difference between U and M is so small that no loss of radiation can occur between them.

Three copper rods, not shown in Fig. 1, terminated by ebonite knobs and soldered to the inside of the outer jacket, served as guides when the inner calorimeter vessel M was let down into the centre of the thermel helix.

With this arrangement and the use of a calibrated Beckmann thermometer for measuring the temperature of the outer bath, heat capacity measurements can be made to the limits of accuracy of the thermometer readings. In the case of the heat capacities of ice over various temperature ranges the authors obtained values reproducible to 0.1 or 0.2% with heat drops in the calorimeter of the order of 1.8°.

In order still further to increase the accuracy of this piece of apparatus the Beckmann thermometer was replaced by a platinum resistance thermometer. This thermometer and resistance bridge together with the galvanometer employed in the later work were those used by Lipsett, Johnson and Maass (8).

With this further improvement the accuracy with which heat capacity measurements could be made was increased to 0.05% or better. In fact the accuracy ultimately obtained was dependent on the means required for transferring the substance under investigation from the initial thermostat bath to the calorimeter.

Method of Procedure

In order to describe the method of manipulating the new calorimeter the following account of a series of determinations of the heat capacities of ice over the temperature range—78.5°C. to 0°C. and the latent heat of fusion at 0°C., is presented.

In this piece of research the apparatus was as described above. a Beckmann thermometer being used to determine the temperature of the outer bath, and consequently that of the inner calorimeter.

A platinum container of about 20 cc. capacity was washed with distilled water, rinsed with alcohol and ether, and then dried to constant weight. About 15 cc. of freshly boiled distilled water was introduced. A platinum screw was inserted in the filling tube in the top of the container and was sealed with DeKhotinsky cement. The platinum container was then placed in a closed brass tube in a thermostat at the desired initial temperature, as described in the paper to which reference has been made (9).

The calorimeter was prepared for use as follows. The inner vessel was weighed empty and dry. Somewhat more than 800 cc. of distilled water at a temperature of about 26°C. was added and the vessel was weighed again. The flange of the copper cover was greased with a known weight of vaseline and the cover was placed on the inner calorimeter vessel. The wires from the inner stirrer were pulled through the two ebonite chimneys (I, I in Fig. 1) and the other chimney (J) was closed with a cork. The closed calorimeter was then placed in the outer jacket (L) and the cover of this vessel was fastened into place. The equipotential shield clamp was screwed on, the outer bath was filled to the overflow with water, and the stirrers in the outer bath were started.

The temperature of the outer bath was kept approximately at that of the inner vessel by a slow drip of hot water until about three-quarters of an hour before the container was introduced.

At this time stirring of the inner calorimeter was commenced. The position of the galvanometer spot when on short circuit was noted and the temperature of the outer bath was brought to exactly the same temperature as that of the inner calorimeter. By regulating the inflow of hot or cold water to the outer bath the galvanometer spot was kept continuously on the zero with the radiation thermel in the circuit. Fine adjustment taps for hot and cold water made it possible to keep the galvanometer spot on the zero for 10 or 15 min. without attention so that ample time was afforded for reading the Beckmann thermometer. Readings of the thermometer were taken every five minutes for one half to three-quarters of an hour.

At the end of this time the container was rapidly introduced in the same manner as previously described (9).

Cold water was run into the outer bath as the inner calorimeter cooled and the temperatures of the two were kept as close together as possible. When equilibrium had been established again the thermometer was read every five minutes for another half to three-quarters of an hour.

At the end of each experiment the outside of the container was carefully dried and the container was placed in a desiccator for several hours. It was then weighed in order to be sure that no leak developed during the test. The calorimeter was taken apart, the inner vessel dried and all vaseline removed with ether.

Beckmann thermometer readings were plotted against time, and the temperature drop in the inner calorimeter when the cold container was introduced was found.

The Beckmann thermometer employed had been standardized by the Bureau of Standards, Washington, and was checked against a standardized platinum resistance thermometer and a second standardized Beckmann thermometer.

A duplicate series of experiments was carried out with the container filled with air.

The results were calculated in the same manner as already described (9). It might be noted that the heat capacities of the container and water were corrected to a final temperature of 25.00°C. from existing data on the specific heats of platinum (11) and water respectively (1).

The water equivalent of the inner calorimeter cover and stirrer was obtained by calculation. For the heat capacity of the ebonite chimneys half the length from the calorimeter cover to the cover of the outer jacket was used. The various specific heat values employed for the various calculations were as follows: ebonite, 0.339 (14); copper, 0.0923 (6); lead, 0.0305 (7); brass, 0.091 (5). The contributions (in calories) of the different parts of the inner calorimeter to the total water equivalent were as follows: chimneys, 0.636; copper cover, 17.438; stirrer (copper weighted with lead), 2.855; inner calorimeter vessel, 24.538. The total water equivalent was 45.48 cal. per degree. The heat capacity of the vaseline on the flanges of the calorimeter cover was less than 0.01 cal. for each experiment and was neglected.

The results obtained are set forth below. Table I shows the heat capacity of the container in cal. per gm. from the initial temperature, given in column 1, to+25.00°C.

TABLE I

HEAT CAPACITIES OF CONTAINER

Initial temperature in deg. C.	Heat capacity in cal. per gm.
+25.0	0.00
0.0	0.766
−26.2	1.614
−50.0	2.331
−78.5	3.159
−78.5	3.160

Since the container was made of platinum, differentiation of the equation representing the heat capacities of the container leads to the following expression for the specific heat of platinum over the temperature range from −78.5°C. to +25.00°C.

$$Cp = 0.013215 + 0.00010148\ T - 0.00000012543\ T^2.$$

Specific heats calculated from this equation agree with those obtained by drawing tangents to the heat capacity curve and some of the values at various temperatures are included in Table II.

TABLE II

SPECIFIC HEATS OF PLATINUM

Temperature in deg. C.	Specific heat in cal. per gm. per deg.
+25.0	0.0323
−10.0	0.0315
−40.0	0.0299
−70.0	0.0284

The value at +25.0°C. agrees exactly with that obtained by Violle (11) for the average specific heat of platinum between 0°C. and 100°C.

The following Table III contains the values obtained for the heat capacity of ice. All the experiments are included in order to show the excellent agreement obtained with the calorimeter for different tests at the same temperatures. In the last column are given the heat capacities at the different temperatures found from a smooth curve drawn through the points in column 3.

TABLE III

HEAT CAPACITIES OF ICE

Experiment	Initial temp. in deg. C.	Heat capacity in cals. per gm.		
		(Observed)	Mean	From smooth curve
1	−3.0	27.93		—
2	−3.0	105.8		105.7
3	−10.0	109.2	109.1	109.2
4	−10.0	108.9		
5	−15.0	111.3	111.4	111.65
6	−15.0	111.4		
7	−20.0	113.8	113.9	114.05
8	−20.0	114.0		
9	−30.0	118.7		118.7
10	−50.0	127.2		127.2
11	−78.5	138.1	138.2	138.2
12	−78.5	138.2		

Table III includes one experiment (No. 1) in which super-cooling of the water occurred. The difference between the heat capacities measured at −3.0°C. and recorded as Experiments 1 and 2 represents the latent heat of fusion of ice at −3.0°C. The value obtained is 77.9 cal. per gm.

The intersection of the heat capacity curve with the temperature ordinate 0°C. gives 104.22 as the heat capacity per gram from 0°C. to +25.00°C. Taking the average specific heat of water between these limits as 1.0020 (1) the heat capacity of the water from 0°C. to +25.00°C. is equal to 25.05 cal. Hence the latent heat of fusion of ice at 0°C.

$$= 104.22 - 25.05$$
$$= 79.2 \text{ cal. per gm.}$$

Discussion

In view of more recent and more accurate determinations of the heat capacities of ice, to be described in a later paper, specific heats calculated from the heat capacity values of Table III are not included here.

An examination of the data presented in Table III shows that not only do the experiments from the various initial temperatures agree to within $\pm 0.15\%$ with one another but also the maximum divergence at $-15.0°$C., from a smooth curve drawn through all the points, is only 0.3%. It thus appears that the probable accuracy of the new adiabatic calorimeter equipped with radiation thermel, and depending on a Beckmann thermometer for measuring the temperature changes taking place, is of the order of 0.2% when this change is of the order of 2°. With a probable error of .001° or .002° in reading the Beckmann this is of the same order of magnitude as the probable error introduced by the Beckmann readings, since two observations are necessary in each experiment. The calorimeter has thus been developed to a stage where the accuracy of the heat measurements obtained with it is dependent on the accuracy with which the temperature of the outer bath, and consequently that of the inner calorimeter, is determined.

The value of 79.2 cal. per gm. for the latent heat of fusion at 0°C. is probably low. Space does not permit of a review of previous values obtained for this constant and, in any case ,they have been collected and discussed elsewhere (2), but the correct value probably lies between 79.4 and 79.5 cal. This involves a probable error of 0.25% to 0.37% in the figure given in this paper, and results from the fact that the heat capacity values from the higher initial temperatures (involving the smallest heat change in the calorimeter) show the greatest divergence from a smooth curve.

The figure of 77.9 cal. per gm. for the latent heat of fusion of ice at $-3.0°$C. is subject to a smaller degree of accuracy because it is dependent on a heat capacity measurement of 27.93 cal. (Experiment 1, Table III) involving a temperature drop of only about 0.5°. It is in agreement however with the Clausius (4) equation $\left(\frac{dL}{dT}\right) = C_w - C_i$ where $\left(\frac{dL}{dT}\right)$ is the rate of change of the latent heat of fusion with temperature, and C_w and C_i are the specific heats of water and ice respectively. Taking the average specific heat of water between 0°C. and $-3°$C. as 1.01 (3) and that of ice as 0.48 (10), $\left(\frac{dL}{dT}\right) = 0.53$ and the latent heat of fusion becomes (79.4-1.6) or 77.8 cal. per gm. at $-3°$C.

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SINKAGE STUDIES. III. CHANGES IN THE WATER-GAS SYSTEM IN LOGS DURING SEASONING AND FLOTATION[1]

By G. W. Scarth[2] and R. Darnley Gibbs[3]

Abstract

Under laboratory conditions a very large fermentative evolution of carbon dioxide takes place in logs during both seasoning and flotation, increasing their gas phase and their buoyancy. The principal changes which take place in the water-gas system of logs during seasoning and flotation have been followed by weight measurements and analyses showing water distribution.

End penetration has been shown to be an important factor in sinkage, at any rate when evaporation from the upper surface of the log is considerable. Bolts, 2 ft. 8 in. long gained, during flotation for 8 months, from two to three times as much water when their ends were unpainted as when they were painted. End penetration, combined with evaporation, was found to create through the log a current of water which helps to transport dissolved gases, and to reduce the volume of the gas bubbles held in the cells. It thus acts doubly towards reducing buoyancy by replacing evaporation loss and by facilitating the escape of the imprisoned gases.

In a preceding paper (7), the seasonal distribution of the water in tree trunks was described in its broader features. Only the volume of "air" or gas was considered; in a given tree, this is reciprocal to that of water. What is known as to the pressure and composition of the gases in trees and of the more intimate relations between the water and gas systems may now be stated briefly.

Gas Pressure in Trees

Scheit (11) maintained that the spaces in the lumina of the conducting elements which are not filled by water are occupied only by water vapor. This view received some support from Sachs and at the present day is maintained by Priestley (personal communication). Yet, before Scheit's time, there was abundant proof of the presence of air, including analyses by Bohm and others, and since then it has frequently been redemonstrated (See Copeland (6)). Since there is air in solution in the tracheal sap, it must come out of solution when the pressure is lowered by transpiration, or when the temperature is raised. It is quite possible that relatively low pressures may be demonstrated to exist in some of the outer annual rings during active transpiration. Whatever significance this may have, however, from the physiological standpoint, the evidence at present is against the endurance of such reduced pressures once the logs are cut, so that it is probably unimportant from the standpoint of log sinkage.

According to the recent work of MacDougal (8), there is free longitudinal transmission of gas pressure in the sapwood during the season of active transpiration. The evidence is that the gas and water exist in the sapwood in

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alternating concentric columns, and that the bordered pits of the tracheids and the end plates of vessels are permeable to a flow of gas under quite low pressure. This is not contrary to Bailey's (1) finding that a pressure of one to several atmospheres is necessary to force air from an air-filled to a water-filled tracheid against the surface tension of the water in the pores of the pit membrane. When water is absent from the cell cavities the pores in the pit membranes may also be free from water, so that there is no surface to exercise a tension.

MacDougal (8) also finds (again when transpiration is active) that gas can be drawn from the outside air into the wood, longitudinally through the wood, and out again by suction. Fifty per cent of the total volume of gas in a tree was drawn through in 48 hr. by a vacuum chamber. The intercellular spaces in the rays provide radial air channels through both wood and bark to the outside, but on anatomical grounds it is difficult to understand how the air can get into these spaces from the xylem elements except by diffusion through water-saturated cell walls. At any rate, the demonstration that air does apparently pass fairly rapidly from the wood to the outside may explain why the pressure of gas in a tree is never found to fall very low. No tree by its own action ever showed in MacDougal's tests a suction of more than 16 mm. Hg on an air-filled manometer.

On the other hand, positive pressures of more than an atmosphere in excess of that of the air outside are recorded. To explain this, MacDougal (8), suggests that the intercellular spaces become injected with water, preventing escape of gas along the rays; but the gas may easily become enveloped with water without that. When positive pressures are developed (namely, in deciduous trees when leafless but with some root activity), there are furthermore no long vertical gas columns in the sapwood. In fact, in spring, birch becomes almost completely filled with water. The hydrostatic pressure compresses and dissolves the included gas bubbles.

As regards pressure, therefore, it may be concluded that in seasons when the water content is rising, especially during fall and spring, the gas in the wood of deciduous trees occurs as bubbles enveloped by water and may have a pressure of as much as two atmospheres: whereas, in seasons of active transpiration the gas system is more extensive, is in freer communication with the outside air and may have a pressure below atmospheric—but only slightly below, as far as is definitely known. Conifers are never subject to such great fluctuations of water content and their gas pressure probably never departs greatly from that of the air.

Composition of the Gas in Trees

Analysis of the gas in trees, dating as far back as 1866, (6) records the fact that it is different from the outside air. Some recent figures from MacDougal (8) may be quoted. These apply to gas extracted into a vacuum chamber. Possibly the enclosed gases which cannot escape may diverge even further from atmospheric proportions. The maximum concentrations of CO_2 deter-

mined were 12.6% in *Pinus radiata*, or about 40 times that of the air; 18.2% in *Populus MacDougali* or 60 times that of the air; and 26% in *Quercus agrifolia* or 90 times that of air. These high concentrations of CO_2 occur apparently at periods of maximum growth and are attributed to active respiration. The minimum occurs in midwinter, namely, 3.5% in pine and 1.4% in poplar and oak. Whereas carbon dioxide is always higher than in the air, oxygen and the sum of oxygen and carbon dioxide together is always lower.

The slow rate of diffusion of gas through wet wood is one of the results brought out in the paper which follows. Original differences in partial pressure from that of the gases in air may thus persist for a long time in cut logs. More than that, they may be intensified, especially in floating logs, as we shall show directly.

The importance of this from the point of view of sinkage follows from the different solubilities of the respective gases and from the diffusion gradients which are set up. Carbon dioxide is 2.8 times as soluble as oxygen at 20° C. and 3.5 times as soluble at 0° C. The replacement of oxygen by carbon dioxide therefore means a more rapid diffusion of gas to the outside.

Gases in Lakes and Streams

The concentration gradient of dissolved gases between the interior of a floating log and the outside, depends on the state of saturation of the external water as well as on the composition and pressure of the internal gases. A great deal of information as to the concentration of oxygen and carbon dioxide in natural waters is given in the reports of various Biological Surveys (2). In summer the surface water of unpolluted lakes is about saturated with oxygen—either slightly over or slightly under 100% saturation—and very low in carbon dioxide, partly owing to the photosynthetic activity of plant life. At the bottom of many lakes, on the other hand, decay of organic matter reverses this state of affairs. Turbulence as of waterfalls, rapids and waves, strikingly assists aëration. Thus, for example, the water in the channel of the Niagara river above the falls has a concentration of oxygen which is only about 80% of its saturation value (as a result of pollution), while immediately below the falls the concentration is 125% of the saturation value. Oversaturation is probably largely colloidal in nature.

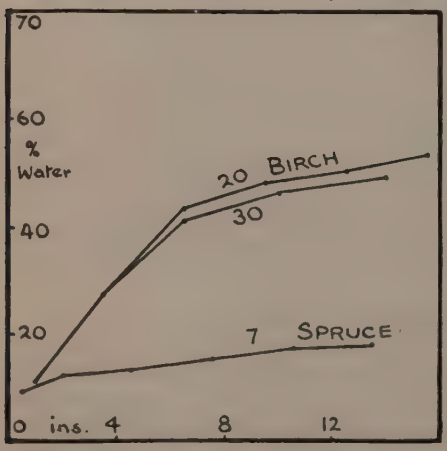

FIG. 1. *Seasoning and end drying of birch and spruce.*

It would seem, therefore, that under ordinary conditions there would be little diffusion gradient of dissolved air between the interior

and exterior of a floating log if the gases inside had the same total and partial pressure as in the atmosphere. Apart, however, from any other causes than higher pressure in the log, the surface tension of the air bubbles adds enough pressure to bring about their gradual dissolution, even in water which contains air in equilibrium with the atmosphere.

Fermentation in Logs: Observations and Experiments

The history of a log during seasoning and floating is not merely one of exchange of gas and water between it and the environment. Under certain conditions there is production of gas in the log and a change of composition of the gases present.

As already noted in an earlier paper (10), Boberg and Juhlin-Dannfelt (4) discovered that floating logs began to lose weight when the temperature of the water rose above 11° C. and found that pieces of wood sank more quickly in water containing $HgCl_2$ than in water which allowed activity of micro-organisms. In the same paper we noted evidence of gas production in storage cells of floating logs in summer. Attention has therefore been given to the question of fermentation in the logs used in the present experiments.

In studying radial sections from the floating logs, the abundance of gas in parenchyma cells, both of the rays and of the longitudinal series, was again a notable feature. Frequently, also, gas extended up and down the tracheids from a ray. The once living cells were usually discolored toward the outside of the log and often filled with granular matter which might be bacterial. In some cases, black bands appear in the wood, resulting from fungal growth.

On applying a strong sodium hydroxide solution to the sections there is an

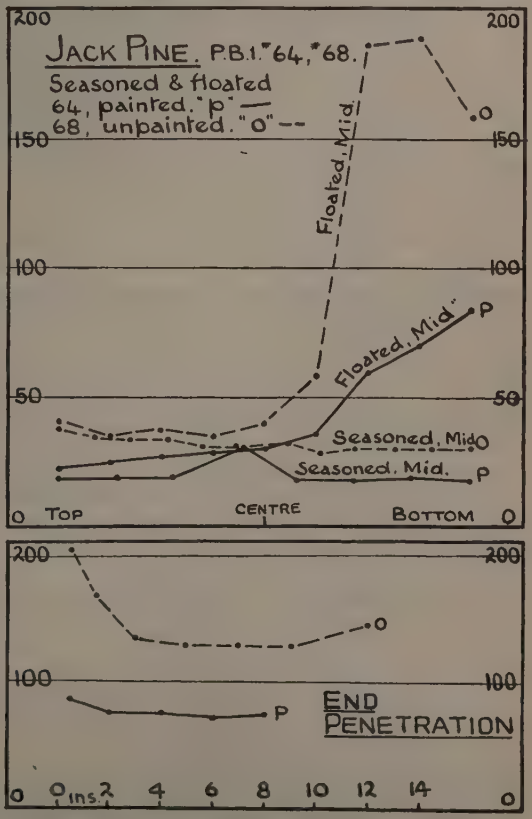

FIG. 2. *Penetration of water into seasoned and floated jack pine.*

immediate and notable shrinkage in the bubbles. Sometimes, in fact, almost all the gas disappears from the rays in the sapwood, indicating that the bulk of it, especially near storage cells, is carbon dioxide. The same condition was found in some of the logs which were seasoning, though they had lain in a warm room for about a year.

To discover roughly how much carbon dioxide was present, bundles of longitudinal strips of wood, 1 or 2 mm. in thickness, were placed in sodium hydroxide in graduate cylinders and similar bundles in water. Generally, the absorption of sodium hydroxide goes on quickly at first and soon comes to a standstill, while that of water goes on at a gradually decreasing rate for many days. When only air is present in the wood, there is very little difference between the early stages of absorption of sodium hydroxide and water respectively. What difference there is develops late rather than early in the process of absorption (see Fig. 4, in the fourth paper of this series which is to be published in this Journal). There is no doubt, therefore, that the difference between the volumes of sodium hydroxide and water, absorbed in the first day, at least represents the volume of carbon dioxide removed—and not all the carbon dioxide present, because the water will dissolve much more of this gas than of oxygen or nitrogen. Table I summarizes the results of the experiments. The proportion of carbon dioxide to total gas is evidently highest when the wood is fairly wet. Toward the upper surface in floating logs, the wood is dry enough to allow direct gaseous diffusion to the outside. The same is true of most of the seasoning wood.

It is clear that these results must not be rashly applied to logs which are exposed to ordinary outdoor conditions. The logs used were at a considerably higher temperature than is experienced by logs in the field, except for a short period in summer. On the other hand, the production of carbon dioxide in the present case is so great that if it is approached at all in the field, even for a few months, the effect must be very important from the standpoint of sinkage. In so far as the carbon dioxide replaces oxygen the change is detrimental to buoyancy owing to the greater solubility of the former gas. In so far as the carbon dioxide is formed by anaërobic fermentation (and it is apparently mainly so) the development is advantageous, adding as it does to the volume and pressure of the gas phase.

Obviously the question of fermentation deserves to be studied further under field conditions.

Seasoning and Flotation Experiments

In continuation of the previous work, the changes in distribution of water during seasoning and flotation were studied in a series of laboratory experiments. It could not be hoped to duplicate field conditions, but conclusions may be applied to these and a number of results have been drawn from material seasoned and floated in the field for purposes of comparison.

TABLE I

CARBON DIOXIDE IN LOGS

Absorption by thin, longitudinal strips of wood of NaOH (about 20%) against that of water.

		Liquid absorbed in per cent. of volume of wood					
		Days					
		¼	1	2	3	4	6
Spruce, 1 yr. seasoned							
Sapwood and heartwood	NaOH	17	24	32	—	—	—
	H_2O	4½	8	12	16	20	23
Sapwood and heartwood	NaOH	2	3	5	—	—	—
	H_2O	1½	2½	4	—	—	—
Birch, 1 yr. seasoned							
Heartwood	NaOH	—	—	35	—	—	—
	H_2O	—	—	26	—	—	—
Spruce, seasoned 4 months, floated 8 months							
Sapwood: Side	NaOH	—	10	13	14	—	—
	H_2O	—	2¼	3¼	4	4½	5
Heartwood	NaOH	—	30	33	33	—	—
	H_2O	—	12	18	21	24	27
Jack Pine, seasoned 4 months, floated 8 months							
Sapwood: Top	NaOH	12	22	—	29	29	—
	H_2O	—	13	—	20	22	—
Heartwood	NaOH	9	15	—	28	30	—
	H_2O	4	12	—	18	20	23
Sapwood: Bottom	NaOH	8	13½	—	14	14½	—
	H_2O	2½	5	—	10	12½	—
Birch, seasoned 4 months, floated 8 months (a) *Unpainted ends: top dry*							
Top	NaOH	—	30	33	36	—	—
	H_2O	—	15	19	20	—	—
Bottom	NaOH	—	17	19	21	—	—
	H_2O	—	4	6½	8½	—	—
(b) *Painted ends: top wetter*							
Top	NaOH	—	23	23	23	—	—
	H_2O	—	6	9	12	—	—
Side	NaOH	—	19	20	20	—	—
	H_2O	—	5	7	9	—	—

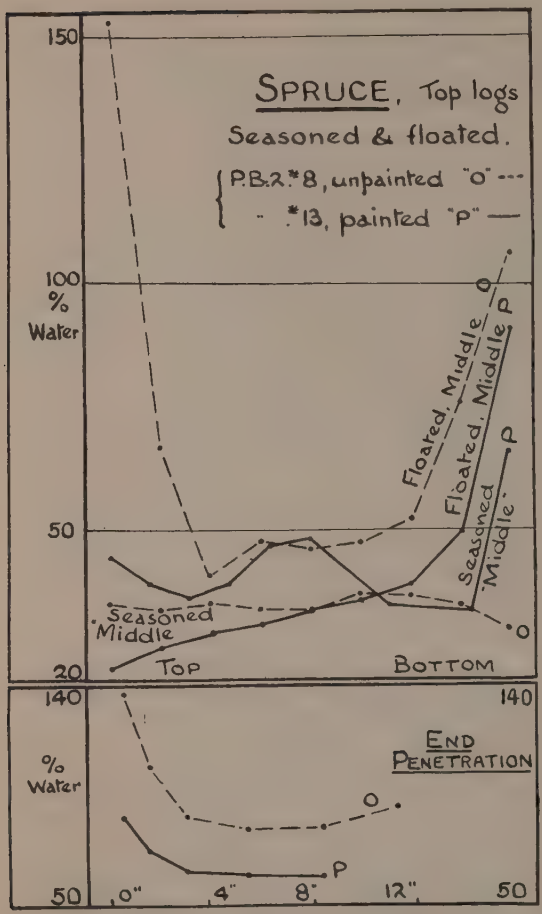

FIG. 3. *Penetration of water into seasoned and floated spruce.*

The study was confined to those types of log which are most liable to sink, namely, coniferous top logs and hardwoods. It is a matter for regret that, even with this restriction, practical considerations limited the choice of logs and curtailed examination to some extent.

The material used consisted of weighed four-foot bolts from lots of which the original (fresh) moisture distribution was known or could be approximated. This material was allowed to season in a fairly well ventilated room during the period extending from April to early August. In August the logs were weighed again to determine the loss during seasoning. At this time, a sample disc was taken over a foot from the end of each and the moisture distribution determined. The logs were cut to uniform length of 31.5 in., reweighed and floated in tanks after the ends of alternate logs of similar pairs had received four coats of Sherwin-Williams "Enamelo"—recommended by the makers as waterproof.

During flotation the logs were disturbed but little and the water, which was changed infrequently, became very foul; the logs themselves having a coat of slime.

In April, 1930, after a period of 7.5 months in the tanks, the logs were reweighed and cut up for the determination of moisture distribution.

As to the seasoning conditions in the room where the logs were stored, the temperature from April to August was more uniform and probably lower on the average than out of doors. There was little checking of the logs and that only for a short distance from the ends.

The amount of end drying was not measured, unfortunately, when the experimental logs were floated. After a year of seasoning the longitudinal distribution in a few logs was determined (Fig. 1). There was still a notable gradient towards the ends in the birch samples but the spruce (small top logs) had become too dry to show much to be observed. In considering the relation of log length to sinkage, the fact of end drying has to be taken into account as well as that of end penetration with which these experiments are more directly concerned.

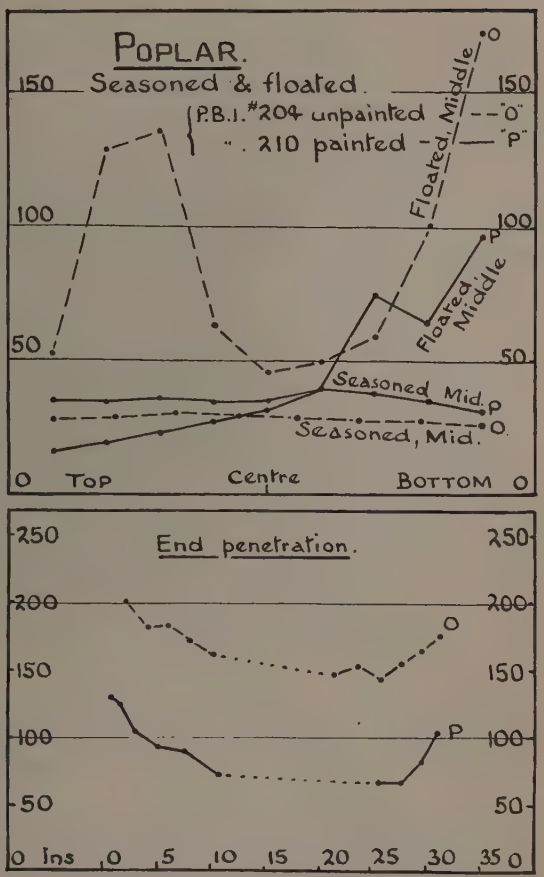

FIG. 4. *Penetration of water into seasoned and floated poplar.*

Weight Changes

Table II summarizes the results of the weighings of whole logs. Seasoning loss and floating gain are given in percentage of seasoned weight, so that they may be compared with one another. Rapid drying and rapid absorption go together, as in poplar and jack pine. The floating gain is also given in pounds, that is, the absolute amount of water added to the logs. Comparing logs with unpainted and painted ends, we see that in every case the amount of water absorbed by the unpainted member of a pair is two or three times that absorbed by the painted one. The greater buoyancy of the painted logs was evident at a glance as they lay in the tank. Some of the unpainted logs were at the point of sinking when they were cut up.

Column 13 in Table II represents the approximate amount of absorption due to lack of paint, namely, the difference between the actual absorption by the unpainted log and its probable absorption if protected by paint.—The latter (Column 12), owing to a slight difference in the size of the logs of a pair, is calculated on the assumption that absorption would be proportional to the absorbing surface.—This "difference" at least is "end penetration". Actually a considerable amount (x) of water penetrated the painted ends, as shown by

TABLE II

WEIGHT CHANGES OF LOGS IN SEASONING AND FLOATING*

1	2	3	4	5	6	7	8	9	10	11	12	13	14
									FLOATING				
Sample No.	Species	Region	Age of tree	Rings in outer inch	Mean diam. in.	Fresh wt. lb.	Seasoning loss, per cent. seasoned wt.	Treatment	Gain % seasoned wt.	Gain in lb.	Probable gain if painted lb.	Difference due to painting lb.	Difference in % of total gain
9	Spruce	Top		26	3.6	20.4	19	P	12	1.4			
15	Spruce	Top		34	4.1	23.2	22	O	26½	3.1	1.6	1.5	60
13	Spruce	Top		42	4.4	26.0	19	P	10	1.5			
8	Spruce	Top		32	4.8	29.6	18	O	26	4.4	1.7	2.7	50
64	J. Pine	Top	59	15	3.6	16.2	44	P	43	3.2			
68	J. Pine	Top	60	14	4.6	24.5	32	O	76	9 4	4.2	5.2	55
23	Birch	Butt	60		8.8	100.1	18	P	9	5.1			
27	Birch	Butt	60		8.1	92.1	15	O	18	9.6	4.6	5.0	70
28	Birch	Middle	60		7.0	73.5	12	P	5	2.1			
24	Birch	Middle	60		7.4	82.3	13½	O	19	8.6	2.2	6.4	74
72	Birch	Top	120		4.4	30.9	16	P	7	1.5			
77	Birch	Top	120		4.0	26.0	12½	O	17	2.7	1.4	1.3	50
210	Poplar	Top	60	14	3.9	21.5	36	P	26½	2.6			
204	Poplar	Top	59	9	3.7	16.8	67	O	90	6.6	2.5	4.1	62

NOTE:—*Cut April 1929; weighed about a week later.*
Seasoned indoors till Aug. 1929. Floated in tank till April 1930.
Length of logs till seasoned, 4 ft.; after sampling, 2 ft. 7 in.
P = painted ends. O = unpainted ends.

**The figures given show the specific differences and effect of painted ends.*

the distribution curves, so that, other things being equal, the difference between the absorption of a log when unpainted and painted respectively is less by the amount x than the absorption through the unpainted ends. "Other things" however, are not quite "equal", somewhat offsetting the above calculation is the fact that the deeper a log sinks, the greater becomes its absorbing surface and the smaller its evaporating surface. Thus in the deeper floater there is a more rapid increase of water in the log, irrespective of end penetration, than in the higher floater, and thus the faster sinker always tends to increase its lead. On the other hand, the unpainted log has the advantage of drying from the segment of the end which is above water level, which may be considerable at the start.

While it is impossible to estimate end penetration exactly from the present results, it is at any rate true that the "difference" given in Columns 13 and 14 of Table II depends entirely on end penetration: it must therefore follow the same order and is probably not less in amount.

In percentage of the total absorption, the difference is, in jack pine 55%, in spruce 50-60%, in birch 50-74% and in poplar 62%. Apparently top logs of conifers with a large proportion of sapwood are not much less permeable to longitudinal flow of water than the hardwoods considered. The number of samples was too small, however, to justify comparison of species.

Moisture Distribution

Turning now to the relative distribution of water, the upper section of Fig. 2-5 show graphically the vertical distribution midway from the ends of the logs when fresh, four months seasoned and eight months floated respectively. In the lower section the horizontal distribution from one or both ends is shown. Not only is the gradient of water content from the end inward much larger and the fall much greater in the unpainted logs, but the general level from end to end is higher. (One birch proved an exception, but this was accounted for by its drier initial condition.)

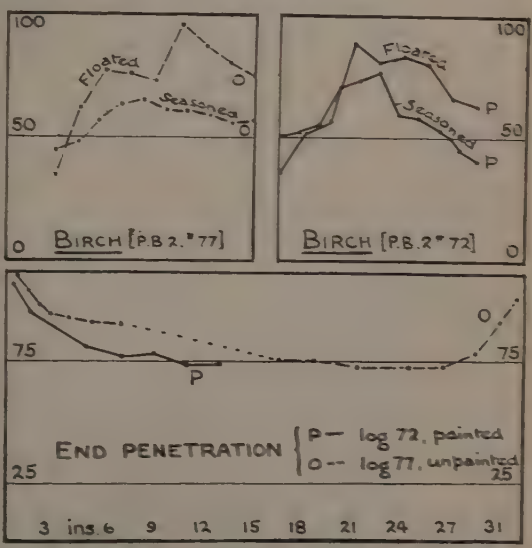

FIG. 5. *Penetration of water into seasoned and floated birch.*

Looking at the vertical distribution and the change therein during that period of flotation, it is seen that the unpainted log in every case has gained much more water on the lower side. On the upper side it has made a large or

small gain, while the painted log has invariably lost water. Fig. 6 and 7 show the vertical distribution across a pair of floating spruce logs at intervals from near the end to the middle. There is very little difference between the end and middle sections in the painted specimen, but a tremendous difference and a big gradation in the unpainted specimen. For about one inch from the end the heartwood is soaked, but beyond that the moisture gradient is practically confined to the sapwood. Thus, the gradient points to a flow through the sapwood from the ends right into the middle region. In birch, the heartwood is penetrated as easily as the sapwood. The structural reasons for this longitudinal permeability were given previously (10).

Evidence of end penetration appears also to the eye when the logs are cut across. There is a progressive expansion of the dry area of the sections from the end inwards: Fig. 8-1 represents this diagrammatically for spruce. Ten inches or more from the end, the wet sapwood does not extend above the water line. Radial penetration does not equal evaporation. Nearer the end it encroaches by tangential penetration because water is supplied faster longitudinally from the ends than evaporation can extract it from the surface. Invasion of the dry heartwood becomes correspondingly greater as the end is approached. Fig. 8-2 represents the middle cut of an unpainted jack pine, and Fig. 8-3 the same cut of an unpainted poplar. They resemble the spruce, but show greater invasion of the heartwood. Fig. 8-4, by way of contrast, shows the middle section of a painted poplar; in this region of the log, evaporation evidently equals or exceeds absorption.

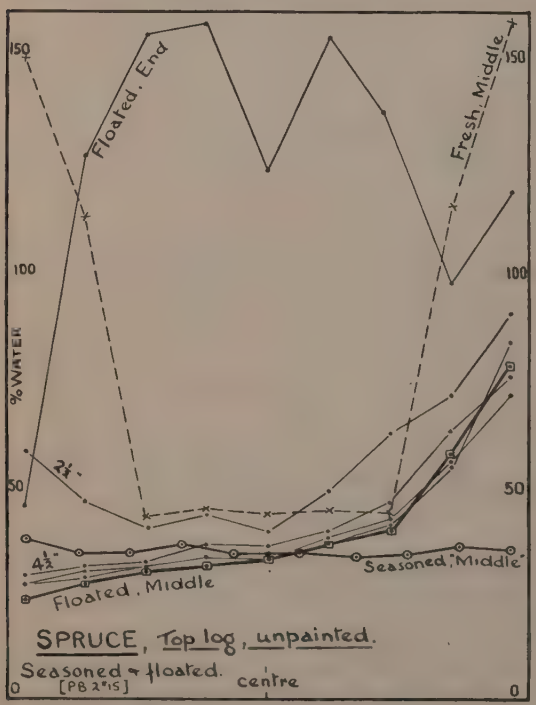

FIG. 6. *Penetration of water into seasoned and floated spruce top log (unpainted).*

It is quite clear from the facts quoted that end penetration is of the greatest importance under the conditions of these experiments.

Escape of Imprisoned Gas

Circulation of water that is set up on the one hand by evaporation and on the other by end and side penetration, is not, however, the essential change

involved in sinkage. Absorption can at most only replace the loss. Sinkage depends on replacement of the gas already in the log by water. Except where the wood is dried to 30% or less of moisture (and that is usually a small section of the log) the same condition exists as was observed for the fresh-cut floating logs (10). Gas bubbles as well as water occupy the cell cavities. They are held there by the walls of the cavities and are not transported by whatever slow current may be passing. So long as there are enough gas bubbles present in it, the log cannot sink though evaporation should cease. Only at the very end of an unpainted log was there any wood completely saturated when the analyses were made. Why do these gas bubbles persist? They are not entirely carbon dioxide formed afresh by fermentation. Since water can enter and flow past them, their disappearance must be limited by the slow rate at which they dissolve. Any current, however gradual, flowing past the gas bubbles should materially assist the slow diffusion of dissolved gas. In point of fact, there is a much smaller proportion of gas in all parts of the unpainted than of the painted logs, the bodily movement of water being greater in the former.

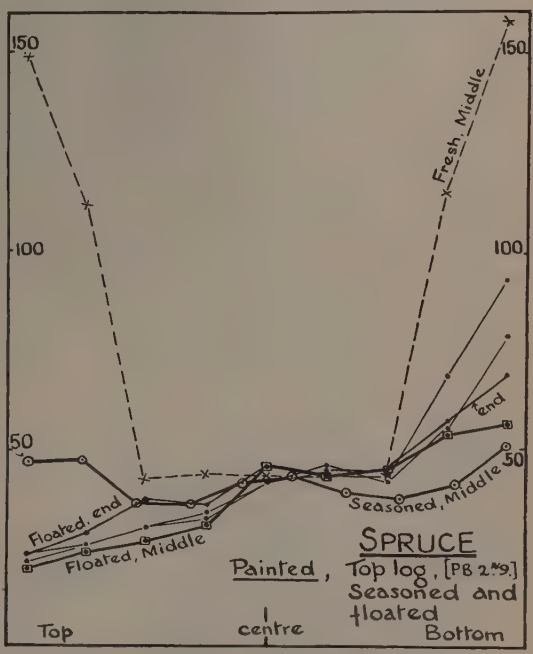

FIG. 7. *Penetration of water into seasoned and floated spruce top log (painted).*

The great bulwark against sinkage is the mass of imprisoned bubbles, whether air or carbon dioxide. Evaporation, by creating a current of water and assisting the solution of the gas, may even be detrimental to flotation unless it actually removes water faster than this can enter the log. In many cases end penetration may prevent this favorable balance.

When the powerful pull due to drying of the cell walls by evaporation is absent or operates only over a small area, the forces which cause water to enter are of a much lower order of magnitude and the rate of absorption is correspondingly slower. The present experiments so far do not yet make it possible to conclude whether the rate of entry under these conditions is directly or indirectly determined by the rate of escape of gas. This question is reserved for a following paper.

Application to Field Conditions

In opposition to the above results certain field tests made in Canada and Sweden have led to the belief that end penetration is not an important factor in sinkage. It must be recognized that there are features in the present experiments which may lead to an abnormal amount of absorption, absolutely and relatively, from the ends. The fact that the bolts were only 2 ft. 8 in. long is easily discounted. Let the side penetration be multiplied by 2, 4 or 5, as the case may be, to give the value for logs of ordinary length, and end penetration is still an important proportion of the whole.

But one feature of the problem of floatability which is not so commonly duplicated in the lakes and rivers is the opportunity for loss of moisture from the exposed surface of the logs. The samples were fairly dry initially and floated in quiet water, in a warm room. In lakes and rivers, logs usually float lower from the start, are liable to be splashed by waves and turned over by currents. Amongst the logs from lakes which have been studied, those most resembling the experimental logs in type of moisture distribution were the air-dried, dead-cut logs, described previously (11). But it seems probable that top drying may often be important in fresh-cut logs which are seasoned longer than usual. This is one of the advantages that seasoning confers. It not only gives greater initial buoyancy, but reduces the net rate of absorption by increasing the surface of evaporation and reducing that of absorption. To get full advantage of this, however, as has been seen, end penetration must be reduced. The shorter the log and also the larger the proportion of sapwood, the more important will this become.

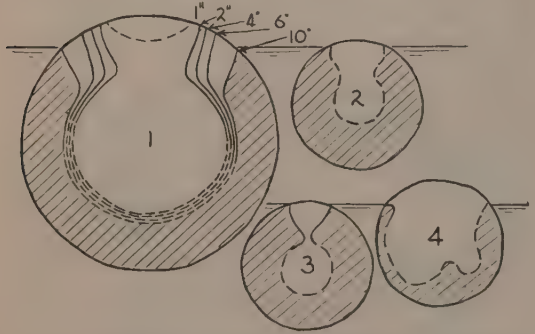

FIG. 8. *Middle cuts showing the progressive expansion of the dry area in (1) unpainted spruce, (2) unpainted jack pine, (3) unpainted poplar, (4) painted poplar.*

On the other hand, end drying is also much faster than side drying per unit surface, and the shorter the log the greater the relative importance of this.

The principal suggestion, therefore, that might be made (on the basis of the above results) toward improving the floatability of birch, poplar, and *even top logs* of softwoods, is that they might be sawn short (say, 4 ft. or so) as soon after cutting as possible, piled with maximum exposure to sun and wind, and have their ends painted or sprayed with a suitable paint or tar just long enough before floating for the paint to dry. Obviously the seasoning interval should be long enough to make this worth while and, as shown in the previous paper (7) the minimum seasoning will be required for hardwoods when they have been cut in the summer.

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